PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/68, G01N 33/574
C07H 21/00

A2

(11) International Publication Number: WO 93/20238

(43) International Publication Date: 14 October 1993 (14.10.93)

(21) International Application Number: PCT/US93/03199

(22) International Filing Date: 7 April 1993 (07.04.93)

(30) Priority data:

867,840	7 April 1992 (07.04.92)	US
903,103	23 June 1992 (23.06.92)	US

(71) Applicant: THE JOHNS HOPKINS UNIVERSITY [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US).

(72) Inventors: BURRELL, Marilee ; 198 Hampshire Street, Cambridge, MA 02139 (US). HILL, David, E. ; 85 Ridge STreet, Arlington, MA 02174 (US). KINZLER, Kenneth, W. ; 1348 Halstead Road, Baltimore, MA 21234 (US). VOGELSTEIN, Bert ; 3700 Breton Way, Baltimore, MA 21208 (US).

(74) Agents: KAGAN, Sarah, A. et al.; Banner, Birch, McKie & Beckett, 1001 G Street, N.W., 11th Floor, Washington, DC 20001-4597 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

(57) Abstract

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to human p53 and allows the cell to escape from p53-regulated growth.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

This application is a continuation-in-part of United States Serial No. 07/903,103, filed June 23, 1992, which is a continuation-in-part of United States Serial No. 07/867,840, filed April 7, 1992, now abandoned.

This invention was made with support from the U.S. Government, including NIH grants CA-57345, CA-43460, CA-02243 and CA-35494. Accordingly, the Government retains certain rights in the invention.

FIELD OF THE INVENTION

The invention relates to the area of cancer diagnostics and therapeutics. More particularly, the invention relates to the detection of a gene which is amplified in certain human tumors.

BACKGROUND OF THE INVENTION

According to the Knudson model for tumorigenesis (Cancer Research, 1985, vol. 45, p. 1482), there are tumor suppressor genes in all normal cells which, when they become non-functional due to mutation, cause neoplastic development. Evidence for this model has been found in cases of retinoblastoma and colorectal tumors. The implicated suppressor genes in these tumors, RB and p53 respectively, were found to be deleted or altered in many of the tumors studied.

The p53 gene product, therefore, appears to be a member of a group of proteins which regulate normal cellular proliferation and suppression of cellular transformation. Mutations in the p53 gene have been linked to tumorigenesis, suggesting that alterations

in p53 protein function are involved in cellular transformation. The inactivation of the p53 gene has been implicated in the genesis or progression of a wide variety of carcinomas (Nigro et al., 1989, *Nature 342*:705-708), including human colorectal carcinoma (Baker et al., 1989, *Science 244*:217-221), human lung cancer (Takahashi et al., 1989, *Science 246*:491-494; Iggo et al., 1990, *Lancet 335*:675-679), chronic myelogenous leukemia (Kelman et al, 1989, *Proc. Natl. Acad. Sci. USA 86*:6783-6787) and osteogenic sarcomas (Masuda et al., 1987, *Proc. Natl. Acad. Sci. USA 84*:7716-7719).

While there exists an enormous body of evidence linking p53 gene mutations to human tumorigenesis (Hollstein et al., 1991, *Science 253*:49-53) little is known about cellular regulators and mediators of p53 function.

Hinds et al. (*Cell Growth & Differentiation, 1*:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated *ras* oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, *Science 253*:49-53. The transformed fibroblasts were found to produce elevated levels of human p53 protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type human p53 gene does not possess transforming activity in cooperation with *ras*. Tuck and Crawford, 1989, *Oncogene Res. 4*:81-96.

Hinds et al., *supra* also expressed human p53 protein in transformed rat cells. When the expressed human p53 was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified M_r 90,000 protein was coimmunoprecipitated. This suggested that the rat M_r 90,000 protein is in a complex with the human p53 protein in the transformed rat cell line.

As mentioned above, levels of p53 protein are often higher in transformed cells than normal cells. This is due to mutations which increase its metabolic stability (Oven et al., 1981, *Mol. Cell. Biol. 1*:101-110; Reich et al. (1983), *Mol. Cell. Biol. 3*:2143-2150). The stabilization of p53 has been associated with complex formation between p53 and viral or cellular proteins. (Linzer and Levine, 1979, *Cell 17*:43-52; Crawford et al., 1981, *Proc. Natl. Acad. Sci. USA 78*:41-45; Dippold et al., 1981, *Proc. Natl. Acad. Sci. USA 78*:1695-1699; Lane and Crawford, 1979, *Nature (Lond.) 278*:261-263; Hinds et al., 1987. *Mol. Cell. Biol. 7*:2863-2869; Finlay et al., 1988, *Mol. Cell. Biol. 8*:531-539; Sarnow et al., 1982, *Cell. 28*:387-394; Gronostajski et al., 1984, *Mol. Cell. Biol. 4*:442-448; Pinhasi-Kimhi et al., 1986, *Nature (Lond.) 320*:182-185; Ruscetti and Scolnick, 1983, *J. Virol. 46*:1022-1026; Pinhasi and Oren, 1984, *Mol. Cell. Biol. 4*:2180-2186; and Sturzbecher et al., 1987, *Oncogene 1*:201-211.) For example, p53 protein has been observed to form oligomeric protein complexes with the SV40 large T antigen, the adenovirus type 5 E1B-M_r 55,000 protein, and the human papilloma virus type 16 or 18 E6 product. Linzer and Levine, 1979, *Cell 17*:43-52; Lane and Crawford, 1979, *Nature, 278*:261-263; Sarnow et al., 1982, *Cell 28*:387-394; Werness et al., 1990, *Science, 248*:76-79. Similarly, complexes have been observed of $p105^{RB}$ (the product of the retinoblastoma susceptibility gene) with T antigen (DeCaprio et al., 1988, *Cell 54*:275-283), the adenovirus E1A protein (Whyte et al., 1988, *Nature 334*:124-129) and the E7 protein of human papilloma virus 16 or 18 (Münger et al., 1989, *EMBO J. 8*:4099-4105). It has been suggested that interactions between these viral proteins and $p105^{RB}$ inactivate a growth-suppressive function of $p105^{RB}$, mimicking deletions and mutations commonly found in the RB gene in tumor cells. In a similar fashion, oligomeric protein complex

formation between these viral proteins and p53 may eliminate or alter the function of p53. Finlay et al., 1989, *Cell 57*:1083-1093.

Fakharzadeh et al. (*EMBO J. 10*:1565-1569, 1991) analyzed amplified DNA sequences present in a tumorigenic mouse cell line (*i.e.*, 3T3DM, a spontaneously transformed derivative of mouse Balb/c cells). Studies were conducted to determine whether any of the amplified genes induced tumorigenicity following introduction of the amplified genes into a nontransformed recipient cell (*e.g.*, mouse NIH3T3 or Rat2 cells). The resulting cell lines were tested for tumorigenicity in nude mice. A gene, designated MDM2, which is amplified more than 50-fold in 3T3DM cells, induced tumorigenicity when overexpressed in NIH3T3 and Rat 2 cells. From the nucleotide and predicted amino acid sequence of mouse MDM2 (mMDM2), Fakharzadeh speculated that this gene encodes a potential DNA binding protein that functions in the modulation of expression of other genes and, when present in excess, interferes with normal constraints on cell growth.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for diagnosing a neoplastic tissue, such as sarcoma, in a human.

It is another object of the invention to provide a cDNA molecule encoding the sequence of human MDM2.

Yet another object of the invention is to provide a preparation of human MDM2 protein which is substantially free of other human cellular proteins.

Still another object of the invention is to provide DNA probes capable of hybridizing with human MDM2 genes or mRNA molecules.

Another object of the invention is to provide antibodies immunoreactive with human MDM2 protein.

Still another object of the invention is to provide kits for detecting amplification or elevated expression of human MDM2.

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method of treating a neoplastic human cell.

Yet another object of the invention is to provide methods for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification.

Still another object of the invention is to provide polypeptides which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method for growing host cells containing a p53 expression vector.

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to human p53 *in vitro*. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C shows the cDNA sequence of human MDM2. In this figure, human and mouse nucleotide and amino acid sequences are compared, the mouse sequence being shown only where it differs from the corresponding human sequence.

Figure 2 shows that hMDM2 binds to p53.

Figure 3 illustrates the amplification of the hMDM2 gene in sarcomas.

Figure 4A-C illustrates hMDM2 expression.

Figure 5 shows the inhibition of p53-mediated transactivation by MDM2. Yeast were stably transfected with expression plasmids encoding p53, lex-VP16, MDM2 or the appropriate vector-only controls, as indicated. p53-responsive (bars a-c) or lexA-responsive (bars d-f) β-galactosidase reporter plasmids were used to assess the response.

Inset: Western blot analysis demonstrating MDM2 (90 kD) and p53 (53 kD) expression in representative yeast strains. The strain indicated by a plus was transfected with expression vector encoding full length MDM2 and p53, while the strain indicated by a minus was transfected only with the p53 expression vector.

Figure 6 shows the determination of MDM2 and p53 domains of interaction. Fig. 5A and Fig. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive β-galactosidase reporter). Yeast clones expressing β-galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined. β-galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. Fig. 6C. Random fragments of p53 were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Figure 7 shows protein expression from the yeast strains described in Figure 6. Western blot analysis was performed as described (Oliner, J.D., et al., *Nature 358*:80-83 (1992)), using 20 μg of protein per lane. The MDM2 and p53 codons contained in the fusion vectors are shown at the top of A and B, respectively. Fig. 7A. Upper panel probed with p53 Ab2 detecting p53; lower panel probed with anti-lexA polyclonal antibodies (lex Ab) detecting MDM2 fusion proteins of 30-50 kD. Fig. 7B. Upper panel probed with Lex Ab detecting the lexA-full length MDM2 fusion protein of 112 kD; lower panel probed with HA Ab (a monoclonal antibody directed against the hemagglutinin epitope tag, Berkeley Antibody) detecting p53 fusion proteins of approximately 25-30 kD.

Figure 8 shows the inhibition of the p53 activation domain by MDM2. Yeast were transfected with expression vectors encoding a lexA-p53 (p53 codons 1-73) fusion (bars a and b) or lexA alone (bar c). Strain b also expressed full length MDM2, and all strains contained the lexA-responsive β-galactosidase reporter plasmid. Inset: Upper panel probed with MDM2 polyclonal antibodies detecting full length MDM2 (90 kD); lower panel probed with lex Ab detecting the lex-p53 fusion protein of 40 kD.

Figure 9 shows a Western blot analysis using monoclonal antibodies to MDM2 or p53. Fifty μg of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis. The position of molecular weight markers, in kd, is given on the right.

Figure 10 demonstrates immunocytochemical analysis of OsA-CL and SW480 cells grown *in vitro*. Monoclonal antibody IF-2, specific for MDM2, and mAb 1801, specific for p53, were used. The exclusively nuclear localization of both proteins is evident, as is the higher expression of MDM2 protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for p53.

Figure 11 demonstrates MDM2 expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for MDM2. Tumors #3 and #10 showed nuclear expression of MDM2, while tumor #2 showed no staining.

DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that a gene exists which is amplified in some human tumors. The amplification of this gene, designated MDM2, is diagnostic of neoplasia or the potential therefor. Detecting the elevated expression of human MDM2-encoded products is also diagnostic of neoplasia or the potential for neoplastic transformation. Over a third of the sarcomas surveyed, including the most common bone and soft tissue forms, were found to have amplified hMDM2 sequences. Expression of hMDM2 was found to be correspondingly elevated in tumors with the gene amplification.

Other genetic alterations leading to elevated hMDM2 expression may be involved in tumorigenesis also, such as mutations in regulatory regions of the gene. Elevated expression of hMDM2 may also be involved in tumors other than sarcomas including but not limited to those in which p53 inactivation has been implicated. These include colorectal carcinoma, lung cancer and chronic myelogenous leukemia.

According to one embodiment of the invention, a method of diagnosing a neoplastic tissue in a human is provided. Tissue or body fluid is isolated from a human, and the copy number of human MDM2 genes is determined. Alternatively, expression levels of human MDM2 gene products can be determined. These include protein and mRNA.

Body fluids which may be tested include urine, serum, blood, feces, saliva, and the like. Tissues suspected of being neoplastic are desirably separated from normal appearing tissue for analysis. This can be done by paraffin or cryostat sectioning or flow cytometry, as is known in the art. Failure to separate neoplastic from non-neoplastic cells can confound the analysis. Adjacent non-neoplastic tissue or any normal tissue can be used to determine a base-line level of expression or copy number, against which the amount of hMDM2 gene or gene products can be compared.

The human MDM2 gene is considered to be amplified if the cell contains more than the normal copy number (2) of this gene per genome. The various techniques for detecting gene amplification are well known in the art. Gene amplification can be determined, for example, by Southern blot analysis, as described in Example 4, wherein cellular DNA from a human tissue is digested, separated, and transferred to a filter where it is hybridized with a probe containing complementary nucleic acids. Alternatively, quantitative polymerase chain reaction (PCR) employing primers can be used to determine gene amplification. Appropriate primers will bind to sequences that bracket human MDM2 coding sequences. Other techniques for determining gene copy number as are known in the art can be used without limitation.

The gene product which is measured may be either mRNA or protein. The term elevated expression means an increase in mRNA production or protein production over that which is normally produced by non-cancerous cells. Although amplification has been observed in human sarcomas, other genetic alterations leading to elevated expression of MDM2 may be present in these or other tumors. Other tumors include those of lung, breast, brain, colorectal, bladder, prostate, liver, skin, and stomach. These, too, are contemplated by the present invention. Non-cancerous cells for use in determining base-line expression levels can be obtained from cells surrounding a tumor, from other humans or from human cell lines. Any increase can have diagnostic value, but generally the mRNA or protein expression will be elevated at least about 3-fold, 5-fold, and in some cases up to about 100-fold over that found in non-cancerous cells. The particular technique employed for detecting mRNA or protein is not critical to the practice of the invention. Increased production of mRNA or protein may be detected, for example, using the techniques of Northern blot analysis or Western blot analysis, respectively, as described in Example 4 or other known techniques such as ELISA, immunoprecipitation, RIA and the like. These techniques are also well known to the skilled artisan.

According to another embodiment of the invention, nucleic acid probes or primers for the determining of human MDM2 gene amplification or elevated expression of mRNA are provided. The probe may comprise ribo- or deoxyribonucleic acids and may contain the entire human MDM2 coding sequence, a sequence complementary thereto, or fragments thereof. A probe may contain, for example, nucleotides 1-949, or 1-2372 as shown in Figure 1. Generally, probes or primers will contain at least about 14 contiguous nucleotides of the human sequence but may desirably contain about 40, 50 or 100 nucleotides. Probes are typically labelled with a fluorescent tag, a radioisotope, or the like to render them easily detectable. Preferably the probes will hybridize under stringent hybridization conditions. Under such conditions they will not hybridize to mouse MDM2. The probes of the invention are complementary to the human MDM2 gene. This means that they share 100% identity with the human sequence.

hMDM2 protein can be produced, according to the invention, substantially free of other human proteins. Provided with the DNA sequence, those of skill in the art can express the cDNA in a non-human cell. Lysates of such cells provide proteins substantially free of other human proteins. The lysates can be further purified, for example, by immunoprecipitation, co-precipitation with p53, or by affinity chromatography.

The antibodies of the invention are specifically reactive with hMDM2 protein. Preferably, they do not cross-react with MDM2 from other species. They can be polyclonal or monoclonal, and can be raised against native hMDM2 or a hMDM2 fusion protein or synthetic peptide. The antibodies are specifically immunoreactive with hMDM2 epitopes which are not present on other human proteins. Some antibodies are reactive with epitopes unique to human MDM2 and not present on the mouse homolog. The antibodies are useful in conventional analyses, such as Western blot analysis, ELISA, immunohistochemistry, and other immunological assays for the detection of proteins. Techniques for raising and purifying polyclonal antibodies are well known in the art, as are techniques for preparing monoclonal antibodies. Antibody binding can be determined by methods known in the art, such as use of an enzyme-labelled secondary antibody, staphylococcal protein A, and the like. Certain monoclonal antibodies of the invention have been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. These include IF2, and ED9, which have been granted accession nos. HB 11290, and HB 11291, respectively.

According to another embodiment of the invention, interference with the expression of MDM2 provides a therapeutic modality. The method can be applied in vivo, in vitro, or ex vivo. For example, expression may be down-regulated by administering triple-strand forming or antisense oligonucleotides which bind to the hMDM2 gene or mRNA, respectively, and prevent transcription or translation. The oligonucleotides may interact with unprocessed pre-mRNA or processed mRNA. Small molecules and peptides which specifically inhibit MDM2 expression can also be used.

Similarly, such molecules which inhibit the binding of MDM2 to p53 would be therapeutic by alleviating the sequestration of p53.

Such inhibitory molecules can be identified by screening for interference of the hMDM2/p53 interaction where one of the binding partners is bound to a solid support and the other partner is labeled. Antibodies specific for epitopes on hMDM2 or p53 which are involved in the binding interaction will interfere with such binding. Solid supports which may be used include any polymers which are known to bind proteins. The support may be in the form of a filter, column packing matrix, beads, and the like. Labeling of proteins can be accomplished according to any technique known in the art. Radiolabels, enzymatic labels, and fluorescent labels can be used advantageously. Alternatively, both hMDM2 and p53 may be in solution and bound molecules separated from unbound subsequently. Any separation technique known in the art may be employed, including immunoprecipitation or immunoaffinity separation with an antibody specific for the unlabeled binding partner.

It has been found that amino acid residues 13-41 of p53 (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and p53. However, additional residues on either the amino or carboxy terminal side of the peptide appear also to be required. Nine to 13 additional p53 residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from p53-regulated growth control in sarcomas, the use of p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53-regulated growth control.

Suitable p53-derived peptides for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

DNA encoding the MDM2-binding, p53-derived peptide, or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus,

or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, *inter alia* encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of peptide, the goal is to alleviate the sequestration of p53 by MDM2.

A cDNA molecule containing the coding sequence of hMDM2 can be used to produce probes and primers. In addition, it can be expressed in cultured cells, such as *E. coli*, to yield preparations of hMDM2 protein substantially free of other human proteins. The proteins produced can be purified, for example, with immunoaffinity techniques using the antibodies described above.

Kits are provided which contain the necessary reagents for determining gene copy number, such as probes or primers specific for the hMDM2 gene, as well as written instructions. The instructions can provide calibration curves to compare with the determined values. Kits are also provided to determine elevated expression of mRNA (*i.e.*, containing probes) or hMDM2 protein (*i.e.*, containing antibodies). Instructions will allow the tester to determine whether the expression levels are elevated. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to human p53. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

The following examples are provided to exemplify various aspects of the invention and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

To obtain human cDNA clones, a cDNA library was screened with a murine MDM2 (mMDM2) cDNA probe. A cDNA library was prepared by using polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 as a template for the production of random hexamer primed double stranded cDNA. Gubler and Hoffmann, 1983, *Gene 25*:263-268. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged, and plated as described by Elledge et al. (*Proc. Natl. Acad. Sci. USA, 88*:1731-1735, 1991). The library was screened initially with a P-labelled (Kinzler, K.W., et al., *Nucl. Acids Res. 17*:3645-3653 (1989), Feinberg and Vogelstein, 1983, *Anal. Biochem. 132*:6-13) mMDM2 cDNA probe (nucleotides 259 to 1508 (Fakharzadeh et al., 1991, *EMBO J. 10*:1565-1569)) and then rescreened with an hMDM2 cDNA clone containing nucleotides 40 to 702.

Twelve clones were obtained, and one of the clones was used to obtain thirteen additional clones by re-screening the same library. In total, twenty-five clones were obtained, partially or totally sequenced, and mapped. Sequence analysis of the twenty-five clones revealed several cDNA forms indicative of alternative splicing. The sequence shown in Figure 1 is representative of the most abundant class and was assembled from three clones: c14-2 (nucleotides 1-949), c89 (nucleotides 467-1737), and c33 (nucleotides 390-2372). The 3' end of the untranslated region has not yet been cloned in mouse or human. The 5' end is likely to be at or near nucleotide 1. There was an open reading frame extending from the 5' end of the human cDNA sequence to nucleotide 1784. Although the signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between hMDM2 and mMDM2 fell off dramatically upstream of nucleotide 312. This lack of conservation in an otherwise highly conserved protein suggested that the sequences upstream of the divergence may not code for protein. Second, an anchored polymerase chain reaction (PCR) approach was employed in an

effort to acquire additional upstream cDNA sequence. Ochman et al., 1985, In: *PCR Technology: Principles and Applications for DNA Amplification* (Erlich, ed.) pp. 105-111 (Stockton, New York). The 5' ends of the PCR derived clones were very similar (within 3 bp) to the 5' ends of clones obtained from the cDNA library, suggesting that the 5' end of the hMDM2 sequence shown in Figure 1 may represent the 5' end of the transcript. Third, *in vitro* translation of the sequence shown in Figure 1, beginning with the methionine encoded by the nucleotide 312 ATG, generated a protein similar in size to that observed in human cells.

In Figure 1, hMDM2 cDNA sequence, hMDM2 and mMDM2 nucleotide and amino acid sequences are compared. The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5' and 3' boundaries of the previously published mMDM2 cDNA. Fakharzadeh et al., 1991, *EMBO J. 10*:1565-1569. Dashes indicate insertions. The mouse and human amino acid sequences are compared from the putative translation start site at nucleotide 312 through the conserved stop codon at nucleotide 1784.

Comparison of the human and mouse MDM2 coding regions revealed significant conservation at the nucleotide (80.3%) and amino acid (80.4%) levels. Although hMDM2 and mMDM2 bore little similarity to other genes recorded in current databases, the two proteins shared several motifs. These included a basic nuclear localization signal (Tanaka, 1990, *FEBS Letters 271*:41-46) at codons 181 to 185, several casein kinase II serine phosphorylation sites (Pinna, 1990, *Biochem. et. Biophys. Acta. 1054*:267-284) at codons 166 to 169, 192 to 195, 269 to 272, and 290 to 293, an acidic activation domain (Ptashne, 1988, *Nature 355*:683-689) at codons 223 to 274, and two metal binding sites (Harrison, 1991, *Nature 353*:715) at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA binding domains. The protein kinase A domain noted in mMDM2 (Fakharzadeh et al., 1991, *EMBO J. 10*:1565-1569) was not conserved in hMDM2.

Example 2

To determine whether the hMDM2 protein could bind to human p53 protein *in vitro*, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Figure 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, *Nucleic Acid Research 15*:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, *Nature Genetics*, in press) and MCC (Kinzler et al., 1991, *Science 251*:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Although the predicted size of the protein generated from the construct was only 55.2 kd (extending from the methionine at nucleotide 312 to nucleotide 1784), *in vitro* translated protein migrated at approximately 95 kilodaltons.

Ten μl of lysate containing the three proteins (hMDM2, p53 and MCC), alone or mixed in pairs, were incubated at 37°C for 15 minutes. One microgram (10 μl) of p53 Ab1 (monoclonal antibody specific for the C-terminus of p53) or Ab2 (monoclonal antibody specific for the N-terminus of p53) (Oncogene Science), or 5 μl of rabbit serum containing MDM2 Ab (polyclonal rabbit anti-hMDM2 antibodies) or preimmune rabbit serum (obtained from the rabbit which produced the hMDM2 Ab), were added as indicated. The polyclonal rabbit antibodies were raised against an *E. coli*-produced hMDM2-glutathione S-transferase fusion protein containing nucleotides 390 to 816 of the hMDM2 cDNA. Ninety μl of RIPA buffer (10 mM tris [pH 7.5], 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer, or Binding Buffer (El-Deriy et al., 1992, *Nature Genetics*, in press) were then added and the mixtures allowed to incubate at 4°C for 2 hours.

Two milligrams of protein A sepharose were added to each tube, and the tubes were rotated end-over-end at 4°C for 1 hour. After pelleting and washing, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and the dried gels autoradiographed for 10 to 60 minutes in the presence of Enhance (New England Nuclear).

Figure 2 shows the co-precipitation of hMDM2 and p53. The three buffers produced similar results, although the co-precipitation was less efficient in SNNTE buffer containing 0.5 M NaCl (Figure 2, lanes 5 and 8) than in Binding Buffer containing 0.1 M NaCl (Figure 2 lanes 6 and 9).

In vitro translated hMDM2, p53 and MCC proteins were mixed as indicated above and incubated with p53 Ab1, p53 Ab2, hMDM2 Ab, or preimmune serum. Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. The bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (Figure 2, lanes 2 and 3). However, when *in vitro* translated human p53 was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association *in vitro* (Figure 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, *Science 251*:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (Figure 2, lanes 8 and 9). When an *in vitro* translated mutant form of p53 (175^{his}) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

In the converse of the experiments described above, the anti-hMDM2 antibodies immunoprecipitated p53 when mixed with hMDM2 protein (Figure 2, lane 15) but failed to precipitate p53 alone (Figure 5, lane 13). Preimmune rabbit serum failed to precipitate either hMDM2 or p53 (Figure 2, lane 16).

Example 3

In order to ascertain the chromosomal localization of hMDM2, somatic cell hybrids were screened with an hMDM2 cDNA probe. A human-hamster hybrid containing only human chromosome 12 was found to hybridize to the probe. Screening of hybrids containing portions of chromosome 12 (Turc-Carel et al., 1986, *Cancer Genet. Cytogenet. 23*:291-299) with the same probe narrowed the localization to chromosome 12q12-14.

Example 4

Previous studies have shown that this region of chromosome 12 is often aberrant in human sarcomas. Mandahl et al., 1987, Genes Chromosomes & Cancer 1:9-14; Turc-Carel et al., 1986, *Cancer Genet. Cytogenet. 23*:291-299; Meltzer et al., 1991, *Cell Growth & Differentiation 2*:495-501. To evaluate the possibility that hMDM2 was genetically altered in such cancers, Southern blot analysis was performed.

Figure 3 shows examples of the amplification of the hMDM2 gene in sarcomas. Cellular DNA (5 μg) was digested with *Eco*RI, separated by agarose gel electrophoresis, and transferred to nylon as described by Reed and Mann (*Nucl. Acids Res., 1985, 13*:7207-7215). The cellular DNA was derived from five primary sarcomas (lanes 1-4, 6) and one sarcoma cell line (OsA-C1, lane 5). The filters were then hybridized with an hMDM2 cDNA fragment probe nucleotide 1-949 (see Figure 1), or to a control probe which identifies fragments of similar size (DCC gene, 1.65 cDNA fragment). Fearon, 1989, *Science 247*:49-56. Hybridization was performed as described by Vogelstein et al. (*Cancer Research, 1987, 47*:4806-4813). A striking amplification of hMDM2 sequences was observed in several of these tumors. (See Figure 3, lanes 2, 3 and 5). Of 47 sarcomas analyzed, 17 exhibited hMDM2 amplification ranging from 5 to 50 fold. These tumors included 7 to 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas (MFH), 3 of 11 osteosarcomas, and 0 and 1 rhabdomyosarcomas. Five benign soft tissue tumors (lipomas) and twenty-seven carcinomas (colorectal or gastric) were also tested by Southern blot analysis and no amplification was observed.

Example 5

This example illustrates that gene amplification is associated with increased expression.

Figure 4A illustrates hMDM2 expression as demonstrated by Northern blot analysis. Because of RNA degradation in the primary sarcomas, only the cell lines could be productively analyzed by Northern blot. RNA was separated by electrophoresis in a MOPS-formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were performed as described by Kinzler et al. (*Nature 332*:371-374, 1988). The RNA was hybridized to the hMDM2 fragment described in Figure 3. Ten μg of total RNA derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains 10 μg of polyadenylated CaCo-2 RNA. RNA sizes are shown in kb. In the one available sarcoma cell line with hMDM2 amplification, a single transcript of approximately 5.5 kb was observed (Figure 4A, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Figure 4A, lane 2) or in a carcinoma cell line (Figure 4A, lane 3). When purified mRNA (rather than total RNA) from the carcinoma cell line was used for analysis, an hMDM2 transcript of 5.5 kb could also be observed (Figure 4A, lane 4).

Figure 4B illustrates hMDM2 expression as demonstrated by Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2).

Figure 4C illustrates hMDM2 expression as demonstrated by Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with hMDM2 amplifications, and lanes 4 and 5 contain protein from sarcomas without hMDM2 amplification.

Western blots using affinity purified MDM2 Ab were performed with 50 μg protein per lane as described by Kinzler et al. (*Mol. Cell. Biol., 1990, 10*:634-642), except that the membranes were blocked in 10% nonfat dried milk and 10% goat serum,

and secondary antibodies were coupled to horseradish peroxidase, permitting chemiluminescent detection (Amersham ECL). MDM2 Ab was affinity purified with a pATH-hMDM2 fusion protein using methods described in Kinzler et al. (*Mol. Cell. Biol. 10*:634-642, 1990). Non-specifically reactive proteins of about 75-85, 105-120 and 170-200 kd were observed in all lanes, irrespective of hMDM2 amplification status. hMDM2 proteins, of about 90-97 kd, were observed only in the hMDM2-amplified tumors. Protein marker sizes are shown in kd.

A protein of approximately 97 kilodaltons was expressed at high levels in the sarcoma cell line with hMDM2 amplification (Figure 4B, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Figure 4B, lanes 1, 2 and 4). Five primary sarcomas were also examined by Western blot analysis. Three primary sarcomas with amplification expressed the same size protein as that observed in the sarcoma cell line (Figure 4C, lanes 1-3), while no protein was observed in the two sarcomas without amplification (Figure 4C, lanes 4 and 5).

Expression of the hMDM2 RNA in the sarcoma with amplification was estimated to be at least 30 fold higher than that in the other lines examined. This was consistent with the results of Western blot analysis.

The above examples demonstrate that hMDM2 binds to p53 *in vitro* and is genetically altered (*i.e.*, amplified) in a significant fraction of sarcomas, including MFH, liposarcomas, and osteosarcomas. These are the most common sarcomas of soft tissue and bone. Weiss and Enzinger, 1978, *Cancer 41*:2250-2266; Malawer et al., 1985, In: *Cancer: Principles and Practice of Oncology*, DeVita et al., Eds., pp. 1293-1342 (Lippincott, Philadelphia).

Human MDM2 amplification is useful for understanding the pathogenesis of these often lethal cancers.

MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as SV40 T-antigen, adenovirus E1B, and HPV E6. Lane and Bechimol, 1990, *Genes and Development 4*:1-8; Werness et al., 1990, *Science*

248:76. Consistent with this hypothesis, no sarcomas with hMDM2 amplification had any of the p53 gene mutations that occur commonly in other tumors. hMDM2 amplification provides a parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. The finding that expression of hMDM2 is correspondingly elevated in tumors with amplification of the gene are consistent with the finding that MDM2 binds to p53, and with the hypothesis that overexpression of MDM2 in sarcomas allows escape from p53 regulated growth control. This mechanism of tumorigenesis has striking parallels to that previously observed for virally induced tumors (Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, *Science 248*:76), in which viral oncogene products bind to and functionally inactivate p53.

Example 6

This example demonstrates that MDM2 expression inhibits p53-mediated transactivation.

To determine if MDM2 could influence the ability of p53 to activate transcription, expression vectors coding for the two proteins were stably transfected into yeast along with a p53-responsive reporter construct. The reporter consisted of a β-galactosidase gene under the transcriptional control of a minimal promoter and a multimerized human DNA sequence which strongly bound p53 *in vitro* (Kern, S.E., et al., *Science 256*:827-830 (1992). Reporter expression was completely dependent on p53 in this assay (Figure 5, compare bars a and c). MDM2 expression was found to inhibit p53-mediated transactivation of this reporter 16-fold relative to isogeneic yeast lacking MDM2 expression (Figure 5, compare bars a and b). Western blot analysis confirmed that p53 (53 kD) was expressed equivalently in strains with and without MDM2 (90 kD) (Figure 1, inset).

METHODS. The MDM2 expression plasmid, pPGK-MDM2, was constructed by inserting the full length MDM2 cDNA (Oliner, J.D., et al., *Nature 358*:80-83 (1992)) into pPGK (Poon, D. *et al., Mol. and Cell.*

Biol. 1111:4809-4821 (1991)), immediately downstream of the phosphoglycerate kinase constitutive promoter. Galactose-inducible p53 (pRS314SN, Nigro, J.M., et al., *Mol. and Cell. Biol. 12*:1357-1365 (1992)), lexA-VP16 (YVLexA, Dalton, S., et al., *Cell 68*:597-612 (1992)), and lexA (YLexA, YVLexA minus VP16) plasmids were used as indicated. The reporters were PG16-*lacZ* (Kern, S.E. *et al., Science 256*:827-830 (1992)) (p53-responsive) and pJK103 (Kamens, J., et al., *Mol. Cell. Biol. 10*:2840-2847 (1990)) (lexA-responsive). *S. cerevisiae* strain pEGY48 was transformed as described (Kinzler, K.W. et al., *Nucl. Acids Res. 17*:3645-3653 (1989)). Yeast strains represented by bars a-c were grown at 30°C to mid-log phase in selective liquid medium containing 2% raffinose as the carbon source, induced for 30 minutes by the addition of 2% galactose, harvested, and lysed as described (Kern, S.E. *et al., Science 256*:827-830 (1992)). The strains represented by bars d-f were treated similarly, except that the cells were induced in galactose for 4 hours to obtain measurable levels of β-galactosidase. β-galactosidase activities shown represent the mean of three to five experimental values (error bars indicate s.e.m.). Protein concentrations were determined by a Coomassie blue-based (bio-Rad) assay. The β-galactosidase assays were performed with AMPGD chemiluminescent substrate and Emerald enhancer (Tropix) according to the manufacturer's instructions. β-galactosidase activities of bars b and c are shown relative to that of bar A; β-galactosidase activities of bars e and f are shown relative to that of bar d. Western blots were performed as described (Oliner, J.D., et al., *Nature 358*:80-83 (1992)), using p53 Ab1801 (lower panel, Oncogene Science) or MDM2 polyclonal antibodies (Oliner, J.D., et al., *Nature 358*:80-83 (1992)) (upper panel).

To ensure that this inhibition was not simply a general transcriptional down regulation mediated by the expression of the foreign MDM2 gene, a yeast strain was created that contained a different transcriptional activator (lexA-VP16, consisting of the lexA DNA binding domain fused to the VP16 acidic activation domain), a similar reporter (with a lexA-responsive site upstream of a β-galactosidase gene), and the same MDM2 expression vector. The results shown in Figure 1 (bars d & e) demonstrate that lexA-VP16 transactivation was unaffected by the presence of MDM2. Furthermore, MDM2 expression had no apparent effect on the growth rate of the cells.

Example 7

This example demonstrates the domains of p53 and MDM2 which interact with each other.

To gain insight into the mechanism of the MDM2-mediated p53 inhibition, the domains of MDM2 and p53 responsible for binding to one another were mapped. The yeast system used to detect protein-protein binding takes advantage of the modular nature of transcription factor domains (Keegan, L., et al., *Science 231*:699-704 (1986); Chien, C.-T., *Proc. Natl. Acad. Sci. U.S.A. 88*:9578-9582 (1991); Brent, R., et al., *Cell 43*:729-731 (1985); Ma, J., et al., *Cell 55*:4430446 (1988). Generically, if protein 1 (fused to a sequence-specific DNA binding domain) is capable of binding to protein 2 (fused to a transcriptional activation domain), then co-expression of both fusion proteins will result in transcriptional activation of a suitable reporter. In our experiments, the lexA DNA binding domain (amino acids 2-202) and the B42 acidic activation domain (AAD) were used in the fusion constructs. The reporter (Kamens, J., et al., *Mol. Cell. Biol. 10*:2840-2847 (1990); contained a lexA-responsive site upstream of a β-galactosidase gene. As an initial control experiment, full length MDM2 was inserted into the lexA fusion vector, and full length p53, supplying its intrinsic activation domain was inserted into a non-fusion vector. The combination resulted in the activation of the lexA-responsive reporter, while the same expression constructs lacking either the MDM2 or p53 cDNA inserts failed to activate β-galactosidase (Table I, strains 1, 2, and 3). Thus, activation was dependent upon MDM2-p53 binding.

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA fragments encoding MDM2 or p53 were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K.W., et al., *Nucl. Acids Res. 17*:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D.M., et al., *Nature 350*:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive β-galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent clones are diagrammed in Fig. 6A. The MDM2 sequences of the remaining 15 p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of Fig. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-terminal to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three p53 sequences shown in Fig. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated fragments.

The resultant yeast colonies were examined for β-galactosidase activity *in situ*. Of approximately 5000 clones containing MDM2 fragments fused to the lexA DNA

binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of β-galactosidase (about 5-fold less than the other fifteen clones) and β-galactosidase expression was independent of p53 expression (Figure 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable β-galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino terminal region of MDM2 (Figure 6B). The β-galactosidase activity of these clones was dependent on p53 co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length p53 contained MDM2 codons 1 to 118 (Figure 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated fragments of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

In a converse set of experiments, yeast clones containing fragments of p53 fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (Figure 2C). The minimal overlap between these three fragments contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the fragments required 9-12 amino acids on either side of codons 13-41 for activity (Figure 6C). To further test the idea that the amino terminal region of p53 was required for MDM2 binding, we generated an additional yeast strain expressing

the lexA-DNA binding domain fused to p53 codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-terminus of p53 were required for the interaction.

TABLE I

STRAIN NUMBER	p53 CONSTRUCT	MDM2 CONSTRUCT	ACTIVATION
1	p53[a]	Vector[b]	-
2	p53[a]	lexA-MDM2[b]	+
3	Vector[a]	lexA-MDM2[b]	-
4	p53[a]	lexA-MDM2 (1-118)[b]	-
5	Vector[a]	lexA-MDM2 (1-118)[b]	-
6	B42-p53 (1-41)[c]	lexA-MDM2[b]	-
7	B42-p53 (1-41)[c]	Vector[b]	-
8	lexA-p53 (74-393)[b]	B42-MDM2[c]	-
9	p53 (1-137)[a]	lexA-MDM2[b]	-

The MDM2 and p53 proteins expressed in each strain, along with the relevant reporters, are indicated. Numbers in parentheses refer to the MDM2 or p53 amino acids encoded (absence of parentheses indicated full length protein, that is, MDM2 amino acids 1 to 491 or p53 amino acids 1 to 393). The lexA-responsive β-galactosidase reporter plasmid (pJK103, Kamens, J., et al., *Mol. Cell. Biol. 10*:2840-2847 (1990)) was present in all strains.

[a]pRS314 vector (Nigro, J.M., et al., *Mol. and Cell. Biol. 12*:1357-1365 (1992).

[b]plex(1-202)+PL vector, containing lexA DNA binding domain fused to insert (Ruden, D.M., et al., *Nature 350*:250-252 (1991).

[c]pJG4-5 vector, containing B42 activation domain fused to insert.

[d](+) indicates that colonies turned blue following 24 hours of incubation on X-gal-containing selective medium, while (-) indicates that colonies remained white following 72 hours of incubation.

Sequence analysis showed that all p53 and MDM2 fragments noted in Figure 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in Figure 6 expressed the relevant proteins at similar levels, as shown by Western blotting (Figure 7).

The most striking results of these mapping experiments was that the region of p53 required to bind MDM2 was almost identical to the previously identified acidic activation domain of p53 (amino acids 20-42) (Unger, T., et al., *EMBO J. 11*:1383-1390 (1992); Miller, C.W., et al., *Proc. Am. Assoc. Cancer Res. 33*:386 (1992). This suggested that MDM2 inhibits p53-mediated transcriptional activation by "concealing" the activation domain of p53 from the transcriptional machinery. If this were true, the p53 activation domain, in isolation from the rest of the p53 protein, should still be inhibitable by full length MDM2. To test this hypothesis, we produced a hybrid protein containing the p53 activation domain (codons 1-73) fused to the lexA-DNA binding domain. This construct exhibited strong transcriptional activation of a lexA-responsive reporter (Figure 8), as predicted from previous experiments in which the p53 activation domain was fused to another DNA binding domain (Fields, S., et al., *Science 249*:1046-1049 (1990); Raycroft, L., et al., *Science 249*:1049-1051 (1990)). The lexA-p53 DNA construct was stably expressed in yeast along with the full length MDM2 expression vector (or the vector alone). MDM2 expression resulted in a five-fold decrease in reporter activity, demonstrating that MDM2 can specifically inhibit the function of the p53 activation domain regardless of the adjacent protein sequences tethering p53 to DNA (Figure 8).

METHODS. Strains were grown to mid-log phase in 2% dextrose before induction of p53 expression for 2 hours by the addition of 2% galactose. The lex-p53 construct was identical to lex-VP16 (YVlexA, Dalton, S., et al., *Cell 68*:597-612 (1992)) except that VP16 sequences were replaced by p53 sequences encoding amino acids 1 to 73.

The results obtained in the experiments discussed herein raise an interesting paradox. If MDM2 binds to (Figure 6) and conceals (Figure 8) the p53 activation

domain from the transcriptional machinery, how could the lexA-MDM2-p53 complex activate transcription from the lexA-responsive reporter (Table I, strain 2)? Because the only functional activation domain in the lexA-MDM2-p53 complex of strain 2 is expected to be contributed by p53, one might predict that it would be concealed by binding to MDM2 and thereby fail to activate. A potential resolution of this paradox is afforded by knowledge that p53 exists as a homotetramer (Stenger, J.E., et al., *Mol. Carcinogenesis* 5:102-106 (1992); Sturzbecher, H.W. *et al., Oncogene* 7:1513-1523 (1992). Thus the activation noted in the lexA-MDM2-p53 complex could be due to the presence of four individual activation domains contributed by the p53 tetramer, not all of which were concealed by MDM2. As a direct test of this issue, the domain of p53 required for homo-oligomerization (Stenger, J.E., et al., *Mol. Carcinogenesis* 5:102-106 (1992); Sturzbecher, H.W. *et al., Oncogene* 7:1513-1523 (1992) (the C-terminus) was removed from the p53 expression construct, so that it consisted of only codons 1-137. This truncated p53 polypeptide retained the entire activation domain (as shown in Figure 8, bar a) and the entire domain required for interaction with MDM2 (Table I, strain 6). Yet, when allowed to interact with lexA-MDM2, no transactivation of the lexA-responsive reporter was observed (Table I, strain 9). Because p53 did not inhibit lexA-MDM2 binding to the lexA reporter (Table I, strain 2), the result of strain 9 is likely to be due to a direct inhibition of the isolated p53 activation domain by MDM2.

Example 8

This example illustrates the production and characterization of antibodies specific for MDM2 epitopes.

The antigen preparations used to intraperitoneally immunize female (BALB/c X C57BL/6)F1 mice comprised bacterially expressed, glutathione-column purified glutathione-S-transferase-MDM2 (GST-MDM2) fusion protein. (One preparation was further purified on a polyacrylamide gel and electroeluted.) The fusion protein contains a 16 kD amino terminal portion of human MDM2 protein (amino acids 27 to

168). For immunization, the fusion protein was mixed with Ribi adjuvant (Ribi Immunochem Research, Inc.).

Two mice were sacrificed and their spleen cells fused to SP2/0s myeloma cells (McKenzie, et al., *Oncogene, 4*:543-548, 1989). Resulting hybridomas were screened by ELISA on trpE-MDM2 fusion protein-coated microtiter wells. The trpE-MDM2 fusion protein contains the same portion of MDM2 as the GST-MDM2 fusion protein. Antigen was coated at a concentration of 1 μg/ml.

A second fusion was performed as described except hybridomas were screened on electroeluted, glutathione purified GST-MDM2. Positive hybridomas from both fusions were expanded and single cell subcloned. Subclones were tested by Western Blot for specificity to the 55 kD trpE-MDM2 and the 43 kD GST-MDM2 fusion proteins.

Two Western Blot positive subclones (1F2 and JG3) were put into mice for ascites generation. The resulting ascites were protein A purified. Both purified monoclonal antibodies tested positive by Western Blot and immunoprecipitation for the 90 kD migrating MDM2 protein present in a human osteosarcoma cell line (OsA-CL), which overexpresses MDM2, and negative in the HOS osteosarcoma, which does not overexpress MDM2.

ED9 was protein G-purified from ascites and found to be specific in cryostat immunohistochemistry for MDM2 in osteosarcoma cells, as was IF2.

Example 9

This example demonstrates the expression and detection of MDM2 at the cellular level.

To evaluate MDM2 expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. (See example 8.) Of several antibodies tested, mAb IF-2 was the most useful, as it detected MDM2 in several assays. For initial testing, we compared proteins derived

from OsA-CL, a sarcoma cell line with MDM2 amplification but without p53 mutation (Table II) and proteins from SW480, a colorectal cancer cell line with p53 mutation (Barak et al., *EMBO 12*:461-468 (1993)) but without MDM2 amplification (data not shown). Figure 9 shows that the mAb IF-2 detected an intense 90 kd band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense 90 kd band in SW480 extracts. We could not distinguish whether the low molecular weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in MDM2 gene copy number in these two lines. Conversely, the 53 kd signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL consistent with the presence of a mutated p53 in SW480 (Fig. 9).

Cells grown on cover slips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 10). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (Barak et al., *EMBO 12*:461-468 (1993)) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186. Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 10), with the relative intensities consistent with the Western blot results (Fig. 9).

The IF-2 mAb was then used (at 5 μg/ml) to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors #3 and #10) stained strongly (Fig. 11). Both of these tumors contained MDM2 gene amplification (Table II). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 11).

TABLE II

TUMOR #	TUMOR ID	TYPE[a]	MDM2 AMPLIFICATION[b]	P53 ALTERATION[c]	OVER-EXPRESSION[d]
1	M-2	MFH	ABSENT	DELETION/ REARRANGEMENT	NONE
2	M-5	MFH	ABSENT	CGC-CUC MUTATION; Arg(158)-His	p53
3	M-7	MFH	PRESENT	NONE OBSERVED	MDM2
4	M-8	MFH	ABSENT	DELETION	NONE
5	M-14	MFH	ABSENT	NONE OBSERVED	N.T.
6	M-15	MFH	ABSENT	DELETION	N.T.
7	M-16	MFH	ABSENT	NONE OBSERVED	NONE
8	M-17	MFH	ABSENT	NONE OBSERVED	N.T.
9	M-18	MFH	ABSENT	OVEREXPRESSED	p53
10	M-20	MFH	PRESENT	NONE OBSERVED	MDM2
11	L-5	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
12	L-7	LIPOSARCOMA	ABSENT	AAC-AGC MUTATION; Asn(239)-Ser	N.T.
13	L-9	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.

TABLE II (Cont.)

TUMOR #	TUMOR ID	TYPE[a]	MDM2 AMPLIFICATION[b]	P53 MUTATION[c]	OVER-EXPRESSION[d]
14	L-11	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
15	KL5B	LIPOSARCOMA	ABSENT	CAG-UAG MUTATION; Gln(144)-Stop	N.T.
16	KL7	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
17	KL10	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
18	KL11	LIPOSARCOMA	ABSENT	GGT-GAT MUTATION; EXON 5 SPLICE DONOR SITE	N.T.
19	KL12	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
20	KL28	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
21	KL30	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
22	S189	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
23	S131B	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
24	OSA-CL	MFH	PRESENT	NONE OBSERVED	MDM2

[a] MFH= malignant fibrous histiocytoma

[b] as assessed by Southern blot

[c] as assessed by Southern blot, sequencing of exons 5-8, or immunohistochemical analysis

[d] as assessed by immunohistochemical analysis; N.T. = not tested

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BURRELL, MARILEE
HILL, DAVID E.
KINZLER, KENNETH W.
VOGELSTEIN, BERT

(ii) TITLE OF INVENTION: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: BANNER, BIRCH, MCKIE AND BECKETT
(B) STREET: 1001 G STREET, N.W.
(C) CITY: WASHINGTON
(D) STATE: D.C.
(E) COUNTRY: USA
(F) ZIP: 20001

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 07-APR-1993

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: KAGAN, SARAH A.

(B) REGISTRATION NUMBER: 32,141

(C) REFERENCE/DOCKET NUMBER: 01107.42798

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 202-508-9100

(B) TELEFAX: 202-508-9299

(C) TELEX: 197430 BBMB UT

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 64 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 17q

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln
1 5 10 15

Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu
20 25 30

Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp
35 40 45

Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro
50 55 60

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2372 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(H) CELL LINE: CaCo-2

(viii) POSITION IN GENOME:

(B) MAP POSITION: 12q12-14

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 312..1784

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGGCC CTGTGTGTCG GAAAGATGGA 60

GCAAGAAGCC GAGCCCGAGG GGCGGCCGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG 120

CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG 180

GAGAGTGGAA TGATCCCCGA GGCCCAGGGC GTCGTGCTTC CGCAGTAGTC AGTCCCCGTG 240

AAGGAAACTG GGGAGTCTTG AGGGACCCCC GACTCCAAGC GCGAAAACCC CGGATGGTGA 300

GGAGCAGGCA A ATG TGC AAT ACC AAC ATG TCT GTA CCT ACT GAT GGT GCT 350
Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala
1 5 10

GTA ACC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACC CTG GTT AGA 398
Val Thr Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg
15 20 25

CCA AAG CCA TTG CTT TTG AAG TTA TTA AAG TCT GTT GGT GCA CAA AAA 446
Pro Lys Pro Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Lys
30 35 40 45

GAC ACT TAT ACT ATG AAA GAG GTT CTT TTT TAT CTT GGC CAG TAT ATT 494
Asp Thr Tyr Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile
50 55 60

ATG ACT AAA CGA TTA TAT GAT GAG AAG CAA CAA CAT ATT GTA TAT TGT 542
Met Thr Lys Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys
65 70 75

TCA AAT GAT CTT CTA GGA GAT TTG TTT GGC GTG CCA AGC TTC TCT GTG 590
Ser Asn Asp Leu Leu Gly Asp Leu Phe Gly Val Pro Ser Phe Ser Val
80 85 90

AAA GAG CAC AGG AAA ATA TAT ACC ATG ATC TAC AGG AAC TTG GTA GTA 638
Lys Glu His Arg Lys Ile Tyr Thr Met Ile Tyr Arg Asn Leu Val Val
95 100 105

GTC AAT CAG CAG GAA TCA TCG GAC TCA GGT ACA TCT GTG AGT GAG AAC 686
Val Asn Gln Gln Glu Ser Ser Asp Ser Gly Thr Ser Val Ser Glu Asn
110 115 120 125

AGG TGT CAC CTT GAA GGT GGG AGT GAT CAA AAG GAC CTT GTA CAA GAG 734
Arg Cys His Leu Glu Gly Gly Ser Asp Gln Lys Asp Leu Val Gln Glu
130 135 140

CTT CAG GAA GAG AAA CCT TCA TCT TCA CAT TTG GTT TCT AGA CCA TCT 782
Leu Gln Glu Glu Lys Pro Ser Ser Ser His Leu Val Ser Arg Pro Ser
145 150 155

ACC TCA TCT AGA AGG AGA GCA ATT AGT GAG ACA GAA GAA AAT TCA GAT 830
Thr Ser Ser Arg Arg Arg Ala Ile Ser Glu Thr Glu Glu Asn Ser Asp
160 165 170

GAA TTA TCT GGT GAA CGA CAA AGA AAA CGC CAC AAA TCT GAT AGT ATT 878
Glu Leu Ser Gly Glu Arg Gln Arg Lys Arg His Lys Ser Asp Ser Ile
175 180 185

TCC CTT TCC TTT GAT GAA AGC CTG GCT CTG TGT GTA ATA AGG GAG ATA 926
Ser Leu Ser Phe Asp Glu Ser Leu Ala Leu Cys Val Ile Arg Glu Ile
190 195 200 205

TGT TGT GAA AGA AGC AGT AGC AGT GAA TCT ACA GGG ACG CCA TCG AAT 974
Cys Cys Glu Arg Ser Ser Ser Ser Glu Ser Thr Gly Thr Pro Ser Asn
210 215 220

CCG GAT CTT GAT GCT GGT GTA AGT GAA CAT TCA GGT GAT TGG TTG GAT 1022
Pro Asp Leu Asp Ala Gly Val Ser Glu His Ser Gly Asp Trp Leu Asp
225 230 235

CAG GAT TCA GTT TCA GAT CAG TTT AGT GTA GAA TTT GAA GTT GAA TCT 1070
Gln Asp Ser Val Ser Asp Gln Phe Ser Val Glu Phe Glu Val Glu Ser
240 245 250

CTC GAC TCA GAA GAT TAT AGC CTT AGT GAA GAA GGA CAA GAA CTC TCA 1118
Leu Asp Ser Glu Asp Tyr Ser Leu Ser Glu Glu Gly Gln Glu Leu Ser
255 260 265

GAT GAA GAT GAT GAG GTA TAT CAA GTT ACT GTG TAT CAG GCA GGG GAG 1166
Asp Glu Asp Asp Glu Val Tyr Gln Val Thr Val Tyr Gln Ala Gly Glu
270 275 280 285

AGT GAT ACA GAT TCA TTT GAA GAA GAT CCT GAA ATT TCC TTA GCT GAC 1214
Ser Asp Thr Asp Ser Phe Glu Glu Asp Pro Glu Ile Ser Leu Ala Asp
290 295 300

TAT TGG AAA TGC ACT TCA TGC AAT GAA ATG AAT CCC CCC CTT CCA TCA 1262
Tyr Trp Lys Cys Thr Ser Cys Asn Glu Met Asn Pro Pro Leu Pro Ser
305 310 315

CAT TGC AAC AGA TGT TGG GCC CTT CGT GAG AAT TGG CTT CCT GAA GAT 1310
His Cys Asn Arg Cys Trp Ala Leu Arg Glu Asn Trp Leu Pro Glu Asp
320 325 330

AAA GGG AAA GAT AAA GGG GAA ATC TCT GAG AAA GCC AAA CTG GAA AAC 1358
Lys Gly Lys Asp Lys Gly Glu Ile Ser Glu Lys Ala Lys Leu Glu Asn
335 340 345

TCA ACA CAA GCT GAA GAG GGC TTT GAT GTT CCT GAT TGT AAA AAA ACT 1406
Ser Thr Gln Ala Glu Glu Gly Phe Asp Val Pro Asp Cys Lys Lys Thr
350 355 360 365

ATA GTG AAT GAT TCC AGA GAG TCA TGT GTT GAG GAA AAT GAT GAT AAA 1454
Ile Val Asn Asp Ser Arg Glu Ser Cys Val Glu Glu Asn Asp Asp Lys
370 375 380

ATT ACA CAA GCT TCA CAA TCA CAA GAA AGT GAA GAC TAT TCT CAG CCA 1502
Ile Thr Gln Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro
385 390 395

TCA ACT TCT AGT AGC ATT ATT TAT AGC AGC CAA GAA GAT GTG AAA GAG 1550
Ser Thr Ser Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu
400 405 410

TTT GAA AGG GAA GAA ACC CAA GAC AAA GAA GAG AGT GTG GAA TCT AGT 1598
Phe Glu Arg Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser
415 420 425

TTG CCC CTT AAT GCC ATT GAA CCT TGT GTG ATT TGT CAA GGT CGA CCT 1646
Leu Pro Leu Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro
430 435 440 445

AAA AAT GGT TGC ATT GTC CAT GGC AAA ACA GGA CAT CTT ATG GCC TGC 1694
Lys Asn Gly Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys
450 455 460

TTT ACA TGT GCA AAG AAG CTA AAG AAA AGG AAT AAG CCC TGC CCA GTA 1742
Phe Thr Cys Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val
465 470 475

TGT AGA CAA CCA ATT CAA ATG ATT GTG CTA ACT TAT TTC CCC 1784
Cys Arg Gln Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro
480 485 490

TAGTTGACCT GTCTATAAGA GAATTATATA TTTCTAACTA TATAACCCTA GGAATTTAGA 1844

CAACCTGAAA TTTATTCACA TATATCAAAG TGAGAAAATG CCTCAATTCA CATAGATTTC 1904

TTCTCTTTAG TATAATTGAC CTACTTTGGT AGTGGAATAG TGAATACTTA CTATAATTTG 1964

ACTTGAATAT GTAGCTCATC CTTTACACCA ACTCCTAATT TTAAATAATT TCTACTCTGT 2024

CTTAAATGAG AAGTACTTGG TTTTTTTTTT CTTAAATATG TATATGACAT TTAAATGTAA 2084

CTTATTATTT TTTTTGAGAC CGAGTCTTGC TCTGTTACCC AGGCTGGAGT GCAGTGGGTG 2144

ATCTTGGCTC ACTGCAAGCT CTGCCCTCCC CGGGTTCGCA CCATTCTCCT GCCTCAGCCT 2204

CCCAATTAGC TTGGCCTACA GTCATCTGCC ACCACACCTG GCTAATTTTT TGTACTTTTA 2264

GTAGAGACAG GGTTTCACCG TGTTAGCCAG GATGGTCTCG ATCTCCTGAC CTCGTGATCC 2324

GCCCACCTCG GCCTCCCAAA GTGCTGGGAT TACAGGCATG AGCCACCG 2372

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 491 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr
1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr
35 40 45

Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys
50 55 60

Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys Ser Asn Asp
65 70 75 80

Leu Leu Gly Asp Leu Phe Gly Val Pro Ser Phe Ser Val Lys Glu His
85 90 95

Arg Lys Ile Tyr Thr Met Ile Tyr Arg Asn Leu Val Val Val Asn Gln
100 105 110

Gln Glu Ser Ser Asp Ser Gly Thr Ser Val Ser Glu Asn Arg Cys His
115 120 125

Leu Glu Gly Gly Ser Asp Gln Lys Asp Leu Val Gln Glu Leu Gln Glu
130 135 140

Glu Lys Pro Ser Ser Ser His Leu Val Ser Arg Pro Ser Thr Ser Ser
145 150 155 160

Arg Arg Arg Ala Ile Ser Glu Thr Glu Glu Asn Ser Asp Glu Leu Ser
165 170 175

Gly Glu Arg Gln Arg Lys Arg His Lys Ser Asp Ser Ile Ser Leu Ser
180 185 190

Phe Asp Glu Ser Leu Ala Leu Cys Val Ile Arg Glu Ile Cys Cys Glu
195 200 205

Arg Ser Ser Ser Ser Glu Ser Thr Gly Thr Pro Ser Asn Pro Asp Leu
210 215 220

Asp Ala Gly Val Ser Glu His Ser Gly Asp Trp Leu Asp Gln Asp Ser
225 230 235 240

Val Ser Asp Gln Phe Ser Val Glu Phe Glu Val Glu Ser Leu Asp Ser
245 250 255

Glu Asp Tyr Ser Leu Ser Glu Glu Gly Gln Glu Leu Ser Asp Glu Asp
260 265 270

Asp Glu Val Tyr Gln Val Thr Val Tyr Gln Ala Gly Glu Ser Asp Thr
275 280 285

Asp Ser Phe Glu Glu Asp Pro Glu Ile Ser Leu Ala Asp Tyr Trp Lys
290 295 300

Cys Thr Ser Cys Asn Glu Met Asn Pro Pro Leu Pro Ser His Cys Asn
305 310 315 320

Arg Cys Trp Ala Leu Arg Glu Asn Trp Leu Pro Glu Asp Lys Gly Lys
325 330 335

Asp Lys Gly Glu Ile Ser Glu Lys Ala Lys Leu Glu Asn Ser Thr Gln
340 345 350

Ala Glu Glu Gly Phe Asp Val Pro Asp Cys Lys Lys Thr Ile Val Asn
355 360 365

Asp Ser Arg Glu Ser Cys Val Glu Glu Asn Asp Asp Lys Ile Thr Gln
370 375 380

Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro Ser Thr Ser
385 390 395 400

Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu Phe Glu Arg
405 410 415

Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser Leu Pro Leu
420 425 430

Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly
435 440 445

Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys Phe Thr Cys
450 455 460

Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln
465 470 475 480

Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro
485 490

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1710 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Mus musculus

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 202..1668

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT 60

CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC 120

TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGA CCCTCTCGGA TCACCGCGCT 180

TCTCCTGCGG CCTCCAGGCC A ATG TGC AAT ACC AAC ATG TCT GTG TCT ACC 231
Met Cys Asn Thr Asn Met Ser Val Ser Thr
1 5 10

GAG GGT GCT GCA AGC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACT 279
Glu Gly Ala Ala Ser Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr
15 20 25

CTG GTT AGA CCA AAA CCA TTG CTT TTG AAG TTG TTA AAG TCC GTT GGA 327
Leu Val Arg Pro Lys Pro Leu Leu Leu Lys Leu Leu Lys Ser Val Gly
30 35 40

GCG CAA AAC GAC ACT TAC ACT ATG AAA GAG ATT ATA TTT TAT ATT GGC 375
Ala Gln Asn Asp Thr Tyr Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly
45 50 55

CAG TAT ATT ATG ACT AAG AGG TTA TAT GAC GAG AAG CAG CAC ATT 423
Gln Tyr Ile Met Thr Lys Arg Leu Tyr Asp Glu Lys Gln Gln His Ile
60 65 70

GTG TAT TGT TCA AAT GAT CTC CTA GGA GAT GTG TTT GGA GTC CCG AGT 471
Val Tyr Cys Ser Asn Asp Leu Leu Gly Asp Val Phe Gly Val Pro Ser
75 80 85 90

TTC TCT GTG AAG GAG CAC AGG AAA ATA TAT GCA ATG ATC TAC AGA AAT 519
Phe Ser Val Lys Glu His Arg Lys Ile Tyr Ala Met Ile Tyr Arg Asn
95 100 105

TTA GTG GCT GTA AGT CAG CAA GAC TCT GGC ACA TCG CTG AGT GAG AGC 567
Leu Val Ala Val Ser Gln Gln Asp Ser Gly Thr Ser Leu Ser Glu Ser
110 115 120

AGA CGT CAG CCT GAA GGT GGG AGT GAT CTG AAG GAT CCT TTG CAA GCG 615
Arg Arg Gln Pro Glu Gly Gly Ser Asp Leu Lys Asp Pro Leu Gln Ala
125 130 135

CCA CCA GAA GAG AAA CCT TCA TCT TCT GAT TTA ATT TCT AGA CTG TCT 663
Pro Pro Glu Glu Lys Pro Ser Ser Ser Asp Leu Ile Ser Arg Leu Ser
140 145 150

ACC TCA TCT AGA AGG AGA TCC ATT AGT GAG ACA GAA GAG AAC ACA GAT 711
Thr Ser Ser Arg Arg Arg Ser Ile Ser Glu Thr Glu Glu Asn Thr Asp
155 160 165 170

GAG CTA CCT GGG GAG CGG CAC CGG AAG CGC CGC AGG TCC CTG TCC TTT 759
Glu Leu Pro Gly Glu Arg His Arg Lys Arg Arg Arg Ser Leu Ser Phe
175 180 185

GAT CCG AGC CTG GGT CTG TGT GAG CTG AGG GAG ATG TGC AGC GGC GGC 807
Asp Pro Ser Leu Gly Leu Cys Glu Leu Arg Glu Met Cys Ser Gly Gly
190 195 200

ACG AGC AGC AGT AGC AGC AGC AGC AGC GAG TCC ACA GAG ACG CCC TCG 855
Thr Ser Ser Ser Ser Ser Ser Ser Ser Glu Ser Thr Glu Thr Pro Ser
205 210 215

CAT CAG GAT CTT GAC GAT GGC GTA AGT GAG CAT TCT GGT GAT TGC CTG 903
His Gln Asp Leu Asp Asp Gly Val Ser Glu His Ser Gly Asp Cys Leu
220 225 230

GAT CAG GAT TCA GTT TCT GAT CAG TTT AGC GTG GAA TTT GAA GTT GAG 951
Asp Gln Asp Ser Val Ser Asp Gln Phe Ser Val Glu Phe Glu Val Glu
235 240 245 250

TCT CTG GAC TCG GAA GAT TAC AGC CTG AGT GAC GAA GGG CAC GAG CTC 999
Ser Leu Asp Ser Glu Asp Tyr Ser Leu Ser Asp Glu Gly His Glu Leu
255 260 265

TCA GAT GAG GAT GAT GAG GTC TAT CGG GTC ACA GTC TAT CAG ACA GGA 1047
Ser Asp Glu Asp Asp Glu Val Tyr Arg Val Thr Val Tyr Gln Thr Gly
270 275 280

GAA AGC GAT ACA GAC TCT TTT GAA GGA GAT CCT GAG ATT TCC TTA GCT 1095
Glu Ser Asp Thr Asp Ser Phe Glu Gly Asp Pro Glu Ile Ser Leu Ala
285 290 295

GAC TAT TGG AAG TGT ACC TCA TGC AAT GAA ATG AAT CCT CCC CTT CCA 1143
Asp Tyr Trp Lys Cys Thr Ser Cys Asn Glu Met Asn Pro Pro Leu Pro
300 305 310

TCA CAC TGC AAA AGA TGC TGG ACC CTT CGT GAG AAC TGG CTT CCA GAC 1191
Ser His Cys Lys Arg Cys Trp Thr Leu Arg Glu Asn Trp Leu Pro Asp
315 320 325 330

GAT AAG GGG AAA GAT AAA GTG GAA ATC TCT GAA AAA GCC AAA CTG GAA 1239
Asp Lys Gly Lys Asp Lys Val Glu Ile Ser Glu Lys Ala Lys Leu Glu
335 340 345

AAC TCA GCT CAG GCA GAA GAA GGC TTG GAT GTG CCT GAT GGC AAA AAG 1287
Asn Ser Ala Gln Ala Glu Glu Gly Leu Asp Val Pro Asp Gly Lys Lys
350 355 360

CTG ACA GAG AAT GAT GCT AAA GAG CCA TGT GCT GAG GAG GAC AGC GAG 1335
Leu Thr Glu Asn Asp Ala Lys Glu Pro Cys Ala Glu Glu Asp Ser Glu
365 370 375

GAG AAG GCC GAA CAG ACG CCC CTG TCC CAG GAG AGT GAC GAC TAT TCC 1383
Glu Lys Ala Glu Gln Thr Pro Leu Ser Gln Glu Ser Asp Asp Tyr Ser
380 385 390

CAA CCA TCG ACT TCC AGC AGC ATT GTT TAT AGC AGC CAA GAA AGC GTG 1431
Gln Pro Ser Thr Ser Ser Ser Ile Val Tyr Ser Ser Gln Glu Ser Val
395 400 405 410

AAA GAG TTG AAG GAG GAA ACG CAG CAC AAA GAC GAG AGT GTG GAA TCT 1479
Lys Glu Leu Lys Glu Glu Thr Gln His Lys Asp Glu Ser Val Glu Ser
415 420 425

AGC TTC TCC CTG AAT GCC ATC GAA CCA TGT GTG ATC TGC CAG GGG CGG 1527
Ser Phe Ser Leu Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg
430 435 440

CCT AAA AAT GGC TGC ATT GTT CAC GGC AAG ACT GGA CAC CTC ATG TCA 1575
Pro Lys Asn Gly Cys Ile Val His Gly Lys Thr Gly His Leu Met Ser
445 450 455

TGT TTC ACG TGT GCA AAG AAG CTA AAA AAA AGA AAC AAG CCC TGC CCA 1623
Cys Phe Thr Cys Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro
460 465 470

GTG TGC AGA CAG CCA ATC CAA ATG ATT GTG CTA AGT TAC TTC AAC 1668
Val Cys Arg Gln Pro Ile Gln Met Ile Val Leu Ser Tyr Phe Asn
475 480 485

TAGCTGACCT GCTCACAAAA ATAGAATTTT ATATTTCTAA CT 1710

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 489 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Cys Asn Thr Asn Met Ser Val Ser Thr Glu Gly Ala Ala Ser Thr
1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Asn Asp Thr Tyr
35 40 45

Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly Gln Tyr Ile Met Thr Lys
50 55 60

Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys Ser Asn Asp
65 70 75 80

Leu Leu Gly Asp Val Phe Gly Val Pro Ser Phe Ser Val Lys Glu His
85 90 95

Arg Lys Ile Tyr Ala Met Ile Tyr Arg Asn Leu Val Ala Val Ser Gln
100 105 110

Gln Asp Ser Gly Thr Ser Leu Ser Glu Ser Arg Arg Gln Pro Glu Gly
115 120 125

Gly Ser Asp Leu Lys Asp Pro Leu Gln Ala Pro Pro Glu Glu Lys Pro
130 135 140

Ser Ser Ser Asp Leu Ile Ser Arg Leu Ser Thr Ser Ser Arg Arg Arg
145 150 155 160

Ser Ile Ser Glu Thr Glu Glu Asn Thr Asp Glu Leu Pro Gly Glu Arg
165 170 175

His Arg Lys Arg Arg Arg Ser Leu Ser Phe Asp Pro Ser Leu Gly Leu
180 185 190

Cys Glu Leu Arg Glu Met Cys Ser Gly Gly Thr Ser Ser Ser Ser Ser
195 200 205

Ser Ser Ser Glu Ser Thr Glu Thr Pro Ser His Gln Asp Leu Asp Asp
210 215 220

Gly Val Ser Glu His Ser Gly Asp Cys Leu Asp Gln Asp Ser Val Ser
225 230 235 240

Asp Gln Phe Ser Val Glu Phe Glu Val Glu Ser Leu Asp Ser Glu Asp
245 250 255

Tyr Ser Leu Ser Asp Glu Gly His Glu Leu Ser Asp Glu Asp Asp Glu
260 265 270

Val Tyr Arg Val Thr Val Tyr Gln Thr Gly Glu Ser Asp Thr Asp Ser
275 280 285

Phe Glu Gly Asp Pro Glu Ile Ser Leu Ala Asp Tyr Trp Lys Cys Thr
290 295 300

Ser Cys Asn Glu Met Asn Pro Pro Leu Pro Ser His Cys Lys Arg Cys
305 310 315 320

Trp Thr Leu Arg Glu Asn Trp Leu Pro Asp Asp Lys Gly Lys Asp Lys
325 330 335

Val Glu Ile Ser Glu Lys Ala Lys Leu Glu Asn Ser Ala Gln Ala Glu
340 345 350

Glu Gly Leu Asp Val Pro Asp Gly Lys Lys Leu Thr Glu Asn Asp Ala
355 360 365

Lys Glu Pro Cys Ala Glu Glu Asp Ser Glu Glu Lys Ala Glu Gln Thr
370 375 380

Pro Leu Ser Gln Glu Ser Asp Asp Tyr Ser Gln Pro Ser Thr Ser Ser
385 390 395 400

Ser Ile Val Tyr Ser Ser Gln Glu Ser Val Lys Glu Leu Lys Glu Glu
405 410 415

Thr Gln His Lys Asp Glu Ser Val Glu Ser Ser Phe Ser Leu Asn Ala
420 425 430

Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly Cys Ile
435 440 445

Val His Gly Lys Thr Gly His Leu Met Ser Cys Phe Thr Cys Ala Lys
450 455 460

Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln Pro Ile
465 470 475 480

Gln Met Ile Val Leu Ser Tyr Phe Asn
485

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 10, line 19 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☒

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
March 11, 1993	HB 11290

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

Hybridoma: IF2

In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

M. Holmes
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 10, line 19 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X] [3]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852, USA

Date of deposit [5]	Accession Number [5]
March 11, 1993	HB 11290

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

IF2 - Hybridoma

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

M. Holmes
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 10, line 19 of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet [ ] [8]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852, USA

Date of deposit [5]	Accession Number [6]
March 11, 1993	HB 11291

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

ED9 - Hybridoma

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [9] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

M. Holmes

(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

CLAIMS

1. A method of diagnosing a neoplastic tissue in a human comprising:

detecting amplification of human MDM2 gene or elevated expression of a human MDM2 gene product in a tissue or body fluid isolated from a human, wherein amplification of the human MDM2 gene or elevated expression of human MDM2 gene product provides a diagnosis of neoplasia or the potential for neoplastic development.

2. The method of claim 1 wherein gene amplification is detected.

3. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being mRNA.

4. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being human MDM2 protein.

5. The method of claim 3 wherein said mRNA is detected by Northern blot analysis by hybridizing mRNA from said tissue to a human MDM2 nucleotide probe.

6. The method of claim 5 wherein the human MDM2 nucleotide probe comprises nucleotides 1-2372 of human MDM2, as shown in Figure 1, or fragments thereof consisting of at least 14 contiguous nucleotides.

7. The method of claim 4 wherein human MDM2 protein is detected by Western Blot analysis by reacting human MDM2 proteins with antibodies which are immunospecific for MDM2 protein.

8. The method of claim 2 wherein the gene amplification is detected using polymerase chain reaction.

9. The method of claim 2 wherein amplification of the human MDM2 gene is detected by Southern blot analysis wherein the human MDM2 gene is hybridized with a nucleotide probe which is complementary to hMDM2 DNA.

10. The method of claim 2 wherein gene amplification is determined by comparing the copy number of hMDM2 in the tissue to the copy number of hMDM2 in a normal tissue of the human.

11. The method of claim 3 wherein elevated expression of mRNA is determined by comparing the amount of hMDM2 mRNA in the tissue to the amount of hMDM2 mRNA in a normal tissue of the human.

12. The method of claim 4 wherein elevated expression of hMDM2 protein is determined by comparing the amount of hMDM2 protein in the tissue to the amount of hMDM2 protein in a normal tissue of the human.

13. The method of claim 2 wherein gene amplification is detected when at least 3-fold more hMDM-2 DNA is observed in the tissue relative to a control sample comprising a normal tissue.

14. The method of claim 3 wherein elevated expression is detected when at least 3-fold more hMDM-2 mRNA is observed in the tissue relative to a control sample comprising a normal tissue.

15. The method of claim 4 wherein elevated expression is detected when at least 3-fold more hMDM2 protein is observed in the tissue relative to a control sample comprising a normal tissue.

16. The method of claim 1 wherein the neoplasia is a sarcoma.

17. The method of claim 16 wherein the sarcoma is a liposarcoma, malignant fibrous histiocytoma, or osteosarcoma.

18. A cDNA molecule comprising nucleotides 1 to 2372, as shown in Figure 1, or fragments thereof, consisting of at least 14 contiguous nucleotides.

19. The cDNA molecule of claim 18 comprising the coding sequence of human MDM2.

20. Human MDM2 protein substantially free of other human proteins.

21. A preparation of antibodies specifically immunoreactive with human MDM2 protein.

22. The preparation of claim 21 wherein the antibodies are monoclonal antibodies.

23. A nucleotide probe comprising a sequence of at least 10 nucleotides which are complementary to nucleotides 1-2372 of human MDM2 gene, as shown in Figure 1.

24. A kit for detecting the amplification of a human MDM2 gene in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said human MDM2 gene under conditions of high stringency, and instructions for determining said amplification.

25. A kit for detecting elevated expression of a human MDM2 mRNA in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said mRNA, and written instructions for determining elevated expression of mRNA.

26. A kit for detecting elevated expression of a human MDM2 protein in a human tissue or body fluid sample comprising MDM2 protein-specific antibodies and written instructions for determining elevated expression of human MDM2 protein.

27. A method of treating a neoplastic cell or a cell having neoplastic potential, comprising:

administering to a cell a therapeutically effective amount of an inhibitory compound which interferes with the expression of human MDM2 gene.

28. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering antisense oligonucleotides.

29. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering triple-strand forming oligonucleotides which interact with DNA.

30. A method for identifying compounds which interfere with the binding of human MDM-2 to human p53, comprising:

binding a predetermined quantity of a first human protein which is detectably labelled to a second human protein;

adding a compound to be tested for its capacity to inhibit binding of said first and second proteins to each other;

determining the quantity of the first human protein which is displaced from or prevented from binding to the second human protein;

wherein the first human protein is MDM-2 and the second human protein is p53 or the first human protein is p53 and the second human protein is MDM-2.

31. The method of claim 30 wherein one of said two human proteins is fixed to a solid support.

32. The method of claim 30 wherein an antibody specifically immunoreactive with said second human protein is used to separate first human protein bound from unbound first human protein.

33. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering a polypeptide to tumor cells which contain a human MDM2 gene amplification, said polyptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

34. The method of claim 33 wherein said polypeptide comprises amino acids 1-41 of p53.

35. The method of claim 33 wherein said polypeptide comprises amino acids 13-57 of p53.

36. The method of claim 33 wherein said polypeptide comprises amino acids 1-50 of p53.

37. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

38. The method of claim 37 wherein said polypeptide comprises amino acids 1-41 of p53.

39. The method of claim 37 wherein said polypeptide comprises amino acids 13-57 of p53.

40. The method of claim 37 wherein said polypeptide comprises amino acids 1-50 of p53.

41. A polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide capable of binding to human MDM2.

42. The polypeptide of claim 41 which comprises amino acids 1-41 of p53.

43. The polypeptide of claim 41 which comprises amino acids 13-57 of p53.

44. The polypeptide of claim 41 which comprises amino acids 1-50 of p53.

45. The preparation of claim 21 wherein the antibodies do not bind to other human proteins.

46. The preparation of claim 21 wherein the antibodies do not bind to human proteins of M_r 75-85K, 105-120K, and 170-200K.

47. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).

48. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma ED9 (ATCC HB 11291).

49. The method of claim 7 wherein the antibodies bind to the epitope on hMDM2 bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).

50. The method of claim 4 wherein human MDM2 protein is detected by immunohistochemistry.

51. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by ED9 (ATCC HB 11291).

52. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by IF2 (ATCC HB 11290).

53. The method of claim 4 wherein human MDM2 protein is detected by immunoprecipitation.

54. A hybridoma cell having the identifying characteristics of ED9 (ATCC HB 11291).

55. A hybridoma cell having the identifying characteristics of IF2 (ATCC HB 11290).

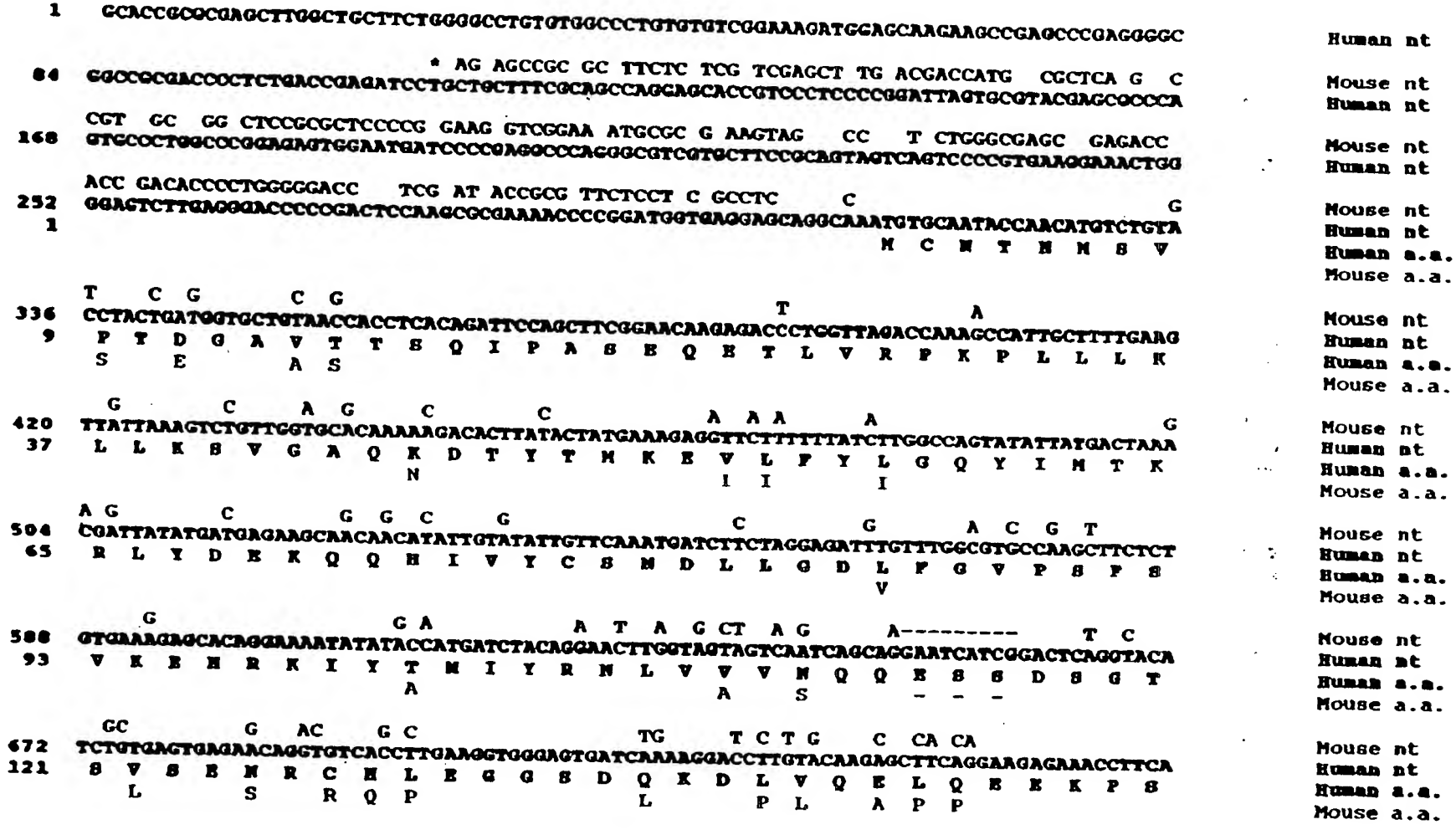

1
GCACCGCGCGAGCTTGGCTGCTTCTGGGGCCTGTGTGGCCCTGTGTGTCGGAAAGATGGAGCAAGAAGCCGAGCCCGAGGGGC
Human nt
* AG AGCCGC GC TTCTC TCG TCGAGCT TG ACGACCATG CGCTCA G C
84
GGCCGCGACCCCTCTGACCGAGATCCTGCTGCTTTCGCAGCCAGCAGCACCGTCCCTCCCCGGATTAGTGCGTACGAGCCCCCA
Mouse nt
Human nt
CGT GC GG CTCCGCGCTCCCCG GAAG GTCGGAA ATGCGC G AAGTAG CC T CTGGGCGAGC GAGACC
168
GTGCCCTGGCCCGCGAGAGTGGAATGATCCCCGAGCCCCAGGGCGTCGTGCTTCCGCAGTAGTCAGTCCCCGTGAAGGAAACTGG
Mouse nt
Human nt
ACC GACACCCCTGGGGGACC TCG AT ACCGCG TTCTCCT C GCCTC C G
252
GGAGTCTTGAGGGACCCCCGACTCCAAGCGCGAAAACCCCGGATGGTGAGGAGCAGGCAAATGTGCAATACCAACATGTCTGTA
1
M C N T N M S V
Mouse nt
Human nt
Human a.a.
Mouse a.a.
T C G C G T A
336
CCTACTGATGGTGCTGTAACCACCTCACAGATTCCAGCTTCGGAACAAGAGACCCTGGTTAGACCAAAGCCATTGCTTTTGAAG
9
P T D G A V T T S Q I P A S E Q E T L V R P K P L L L K
S E A S
Mouse nt
Human nt
Human a.a.
Mouse a.a.
G C A G C C A A A A G
420
TTATTAAAGTCTGTTGGTGCACAAAAAGACACTTATACTATGAAAGAGGTTCTTTTTTATCTTGGCCAGTATATTATGACTAAA
37
L L K S V G A Q K D T Y T M K E V L F Y L G Q Y I M T K
N I I I
Mouse nt
Human nt
Human a.a.
Mouse a.a.
A G C G G C G C A C G T
504
CGATTATATGATGAGAAGCAACAACATATTGTATATTGTTCAAATGATCTTCTAGGAGATTTGTTTGGCGTGCCAAGCTTCTCT
65
R L Y D E K Q Q H I V Y C S M D L L G D L F G V P S F S
V
Mouse nt
Human nt
Human a.a.
Mouse a.a.
G G A A T A G CT A G A---------- T C
588
GTGAAAGAGCACAGGAAAATATATACCATGATCTACAGGAACTTGGTAGTAGTCAATCAGCAGGAATCATCGGACTCAGGTACA
93
V K E H R K I Y T M I Y R N L V V V N Q Q E S S D S G T
A A S - - -
Mouse nt
Human nt
Human a.a.
Mouse a.a.
GC G AC G C TG T C T G C CA CA
672
TCTGTGAGTGAGAACAGGTGTCACCTTGAAGGTGGGAGTGATCAAAAGGACCTTGTACAAGAGCTTCAGGAAGAGAAACCTTCA
121
S V S E N R C H L E G G S D Q K D L V Q E L Q E E K P S
L S R Q P L P L A P P
Mouse nt
Human nt
Human a.a.
Mouse a.a.

FIGURE 1A

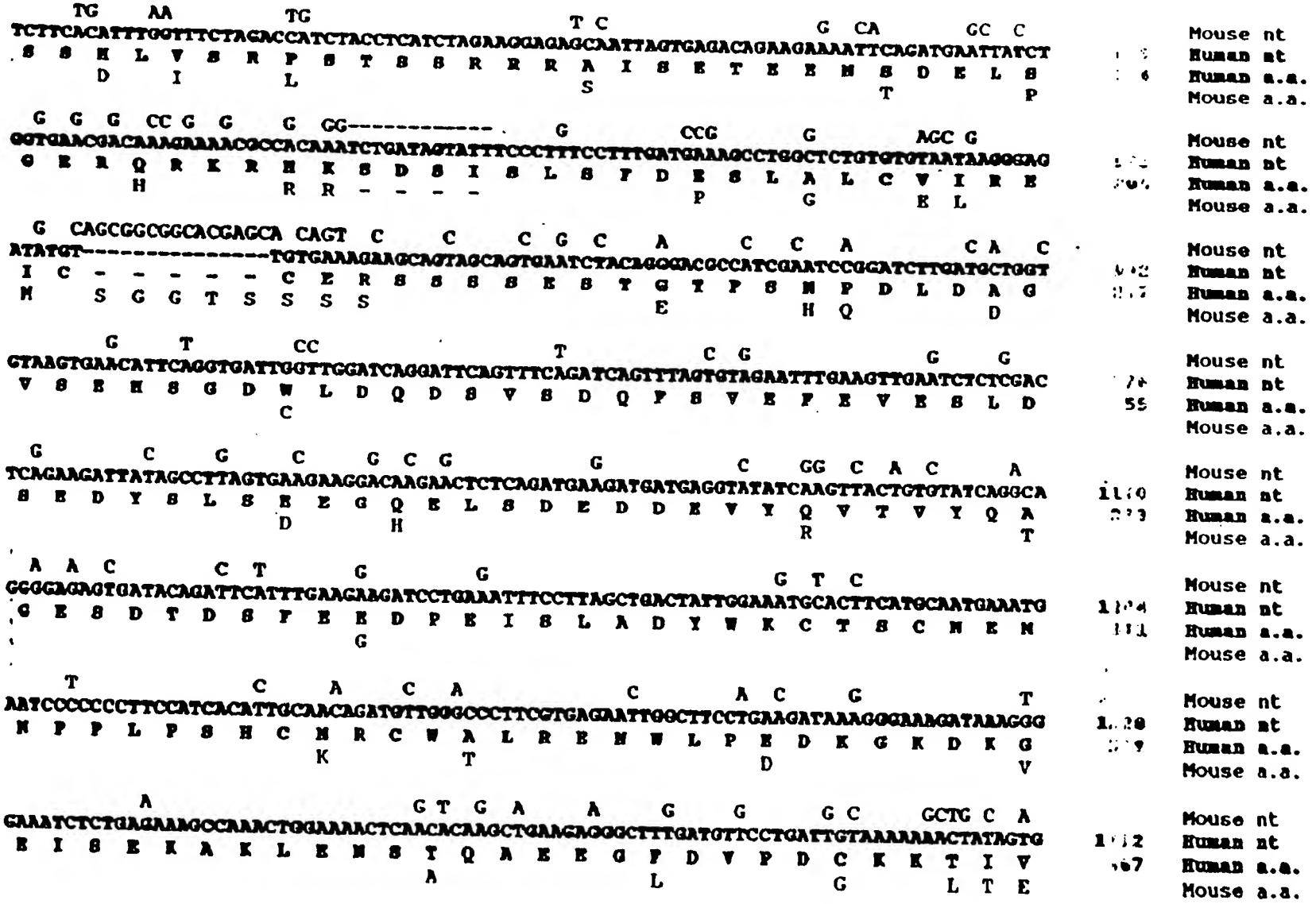


FIGURE 1B

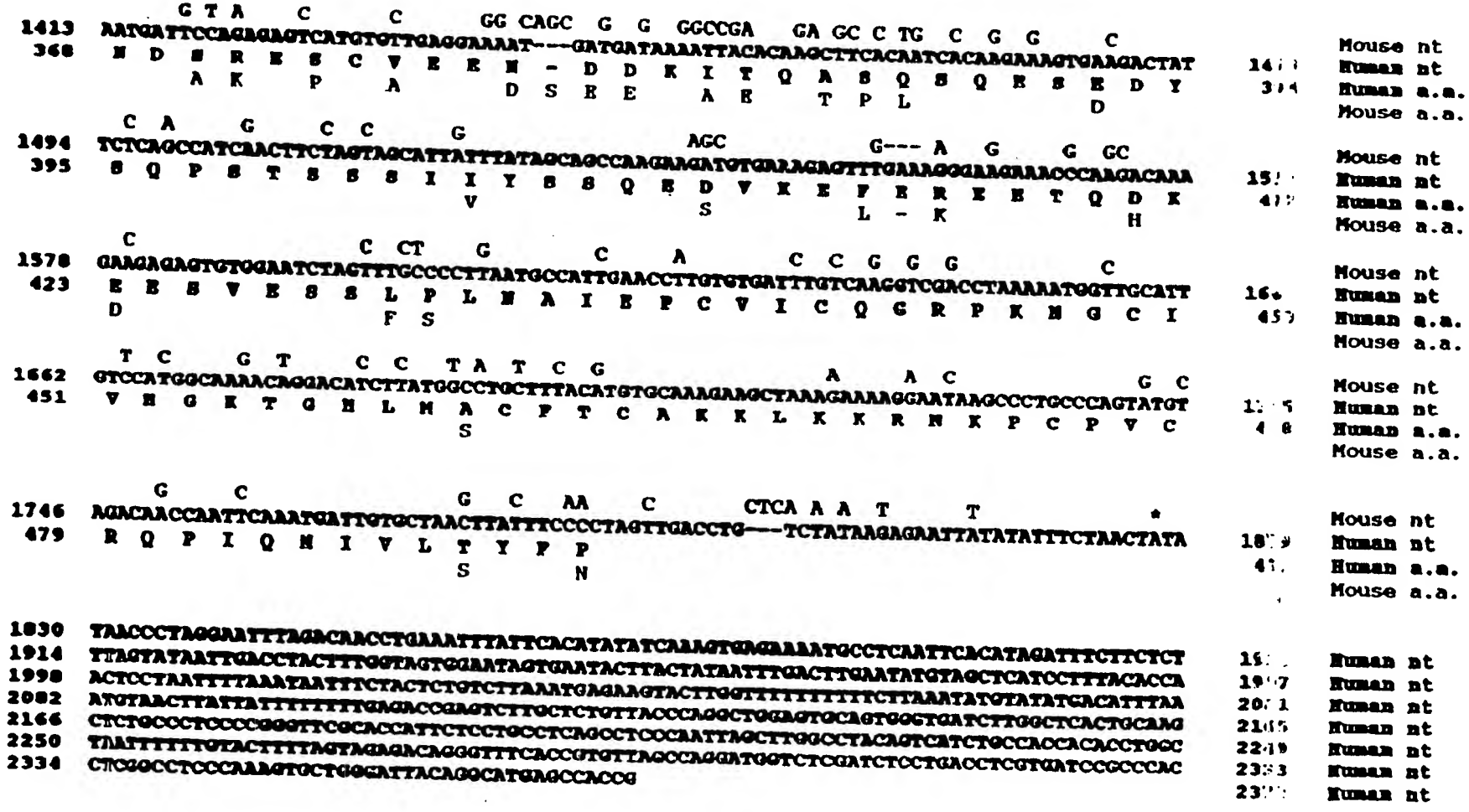


FIGURE 1C

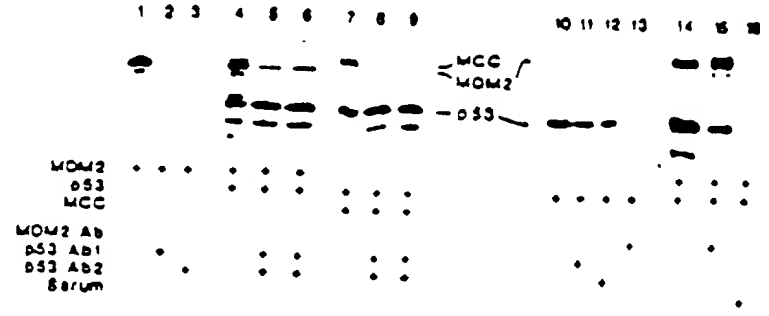

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
MCC
MDM2
p53
MDM2
p53
MCC
MDM2 Ab
p53 Ab1
p53 Ab2
Serum

FIGURE 2

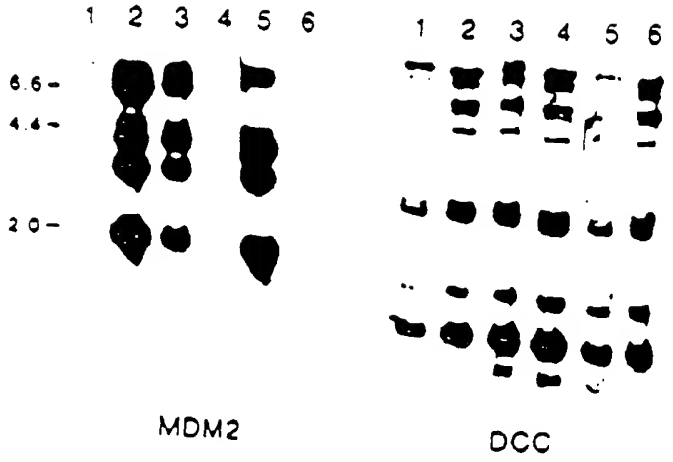


FIGURE 3

FIGURE 4

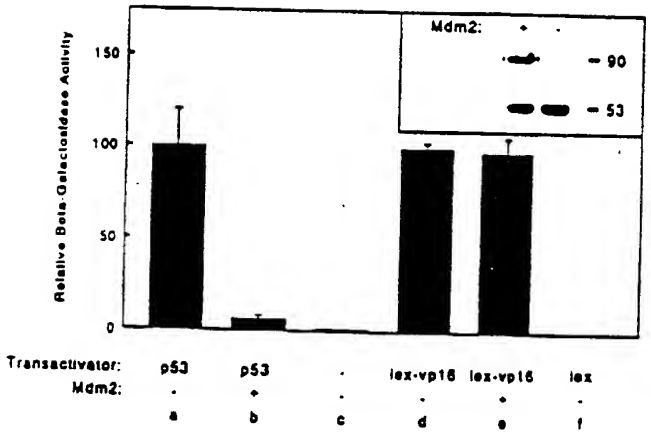

Relative Beta-Galactosidase Activity
150
100
50
0
Mdm2:
+
-
90
53
Transactivator:
Mdm2:
p53
p53
-
lex-vp16
lex-vp16
lex
-
+
-
-
+
-
a
b
c
d
e
f

FIGURE 5

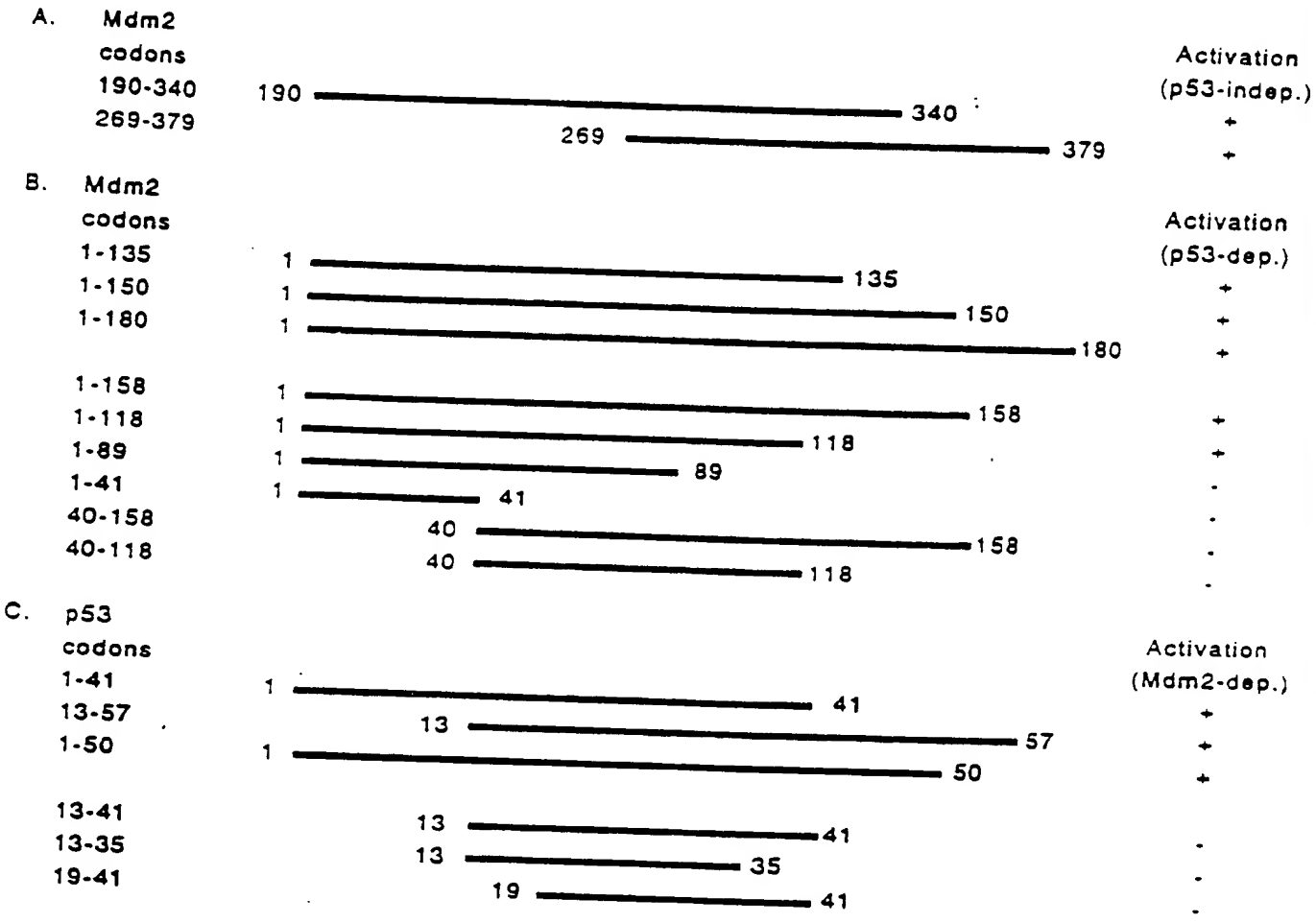

A.
Mdm2 codons
190-340
269-379
Activation (p53-indep.)
190
340
269
379
+
+
B.
Mdm2 codons
1-135
1-150
1-180
1-158
1-118
1-89
1-41
40-158
40-118
Activation (p53-dep.)
1
135
150
180
158
118
89
41
40
+
+
+
+
+
-
-
-
-
C.
p53 codons
1-41
13-57
1-50
13-41
13-35
19-41
Activation (Mdm2-dep.)
1
41
13
57
50
35
+
+
+
-
-
-

FIGURE 6

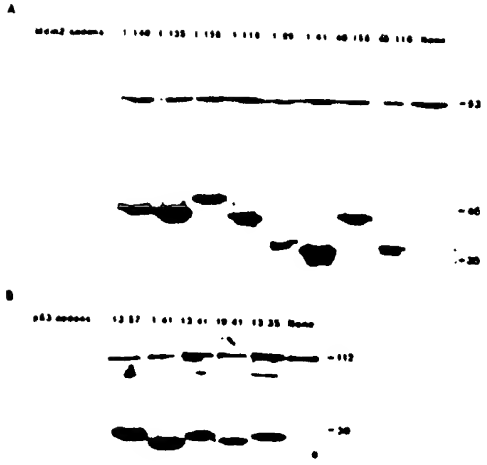


FIGURE 7

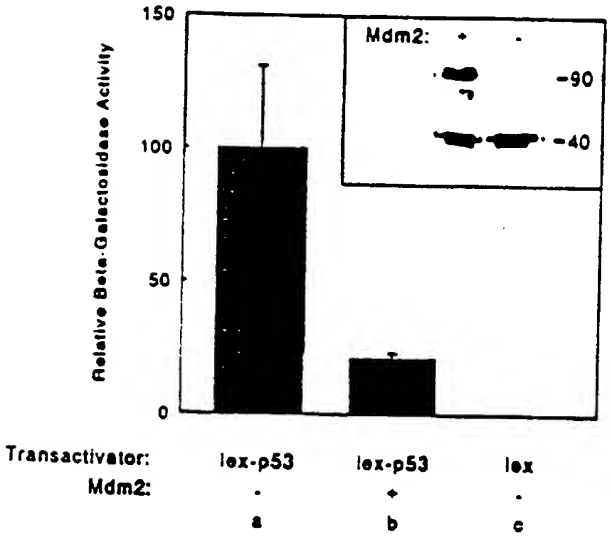


FIGURE 8

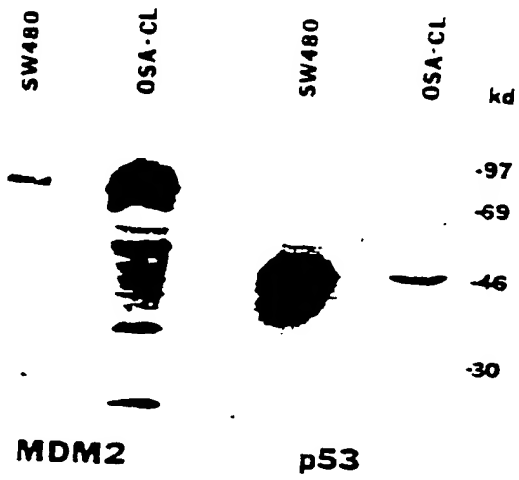


FIGURE 9

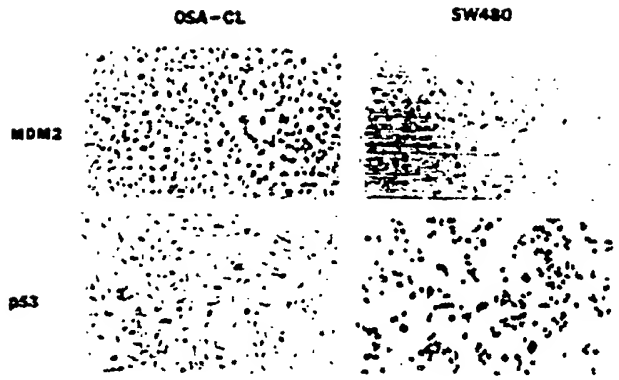


FIGURE 10

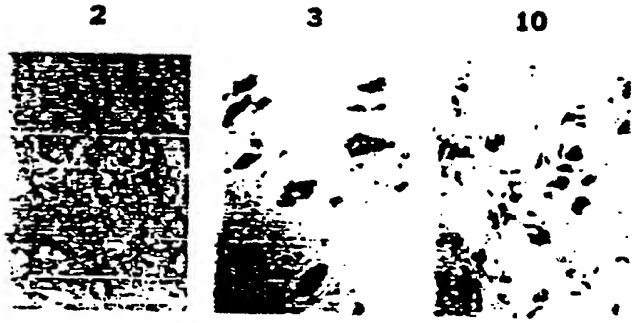


FIGURE 11

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/68, G01N 33/574
C07H 21/00

A3

(11) International Publication Number: WO 93/20238

(43) International Publication Date: 14 October 1993 (14.10.93)

(21) International Application Number: PCT/US93/03199

(22) International Filing Date: 7 April 1993 (07.04.93)

(30) Priority data:

867,840	7 April 1992 (07.04.92)	US
903,103	23 June 1992 (23.06.92)	US

(71) Applicant: THE JOHNS HOPKINS UNIVERSITY [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US).

(72) Inventors: BURRELL, Marilee ; 198 Hampshire Street, Cambridge, MA 02139 (US). HILL, David, E. ; 85 Ridge STreet, Arlington, MA 02174 (US). KINZLER, Kenneth, W. ; 1348 Halstead Road, Baltimore, MA 21234 (US). VOGELSTEIN, Bert ; 3700 Breton Way, Baltimore, MA 21208 (US).

(74) Agents: KAGAN, Sarah, A. et al.; Banner, Birch, McKie & Beckett, 1001 G Street, N.W., 11th Floor, Washington, DC 20001-4597 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
11 November 1993 (11.11.93)

(54) Title: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

(57) Abstract

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to human p53 and allows the cell to escape from p53-regulated growth.

* (Referred to in PCT Gazette No. 29/1993, Section II)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

This application is a continuation-in-part of United States Serial No. 07/903,103, filed June 23, 1992, which is a continuation-in-part of United States Serial No. 07/867,840, filed April 7, 1992, now abandoned.

This invention was made with support from the U.S. Government, including NIH grants CA-57345, CA-43460, CA-02243 and CA-35494. Accordingly, the Government retains certain rights in the invention.

FIELD OF THE INVENTION

The invention relates to the area of cancer diagnostics and therapeutics. More particularly, the invention relates to the detection of a gene which is amplified in certain human tumors.

BACKGROUND OF THE INVENTION

According to the Knudson model for tumorigenesis (Cancer Research, 1985, vol. 45, p. 1482), there are tumor suppressor genes in all normal cells which, when they become non-functional due to mutation, cause neoplastic development. Evidence for this model has been found in cases of retinoblastoma and colorectal tumors. The implicated suppressor genes in these tumors, RB and p53 respectively, were found to be deleted or altered in many of the tumors studied.

The p53 gene product, therefore, appears to be a member of a group of proteins which regulate normal cellular proliferation and suppression of cellular transformation. Mutations in the p53 gene have been linked to tumorigenesis, suggesting that alterations

in p53 protein function are involved in cellular transformation. The inactivation of the p53 gene has been implicated in the genesis or progression of a wide variety of carcinomas (Nigro et al., 1989, *Nature 342*:705-708), including human colorectal carcinoma (Baker et al., 1989, *Science 244*:217-221), human lung cancer (Takahashi et al., 1989, *Science 246*:491-494; Iggo et al., 1990, *Lancet 335*:675-679), chronic myelogenous leukemia (Kelman et al, 1989, *Proc. Natl. Acad. Sci. USA 86*:6783-6787) and osteogenic sarcomas (Masuda et al., 1987, *Proc. Natl. Acad. Sci. USA 84*:7716-7719).

While there exists an enormous body of evidence linking p53 gene mutations to human tumorigenesis (Hollstein et al., 1991, *Science 253*:49-53) little is known about cellular regulators and mediators of p53 function.

Hinds et al. (*Cell Growth & Differentiation, 1*:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated *ras* oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, *Science 253*:49-53. The transformed fibroblasts were found to produce elevated levels of human p53 protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type human p53 gene does not possess transforming activity in cooperation with *ras*. Tuck and Crawford, 1989, *Oncogene Res. 4*:81-96.

Hinds et al., *supra* also expressed human p53 protein in transformed rat cells. When the expressed human p53 was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified M_r 90,000 protein was coimmunoprecipitated. This suggested that the rat M_r 90,000 protein is in a complex with the human p53 protein in the transformed rat cell line.

As mentioned above, levels of p53 protein are often higher in transformed cells than normal cells. This is due to mutations which increase its metabolic stability (Oven et al., 1981, *Mol. Cell. Biol. 1*:101-110; Reich et al. (1983), *Mol. Cell. Biol. 3*:2143-2150). The stabilization of p53 has been associated with complex formation between p53 and viral or cellular proteins. (Linzer and Levine, 1979, *Cell 17*:43-52; Crawford et al., 1981, *Proc. Natl. Acad. Sci. USA 78*:41-45; Dippold et al., 1981, *Proc. Natl. Acad. Sci. USA 78*:1695-1699; Lane and Crawford, 1979, *Nature (Lond.) 278*:261-263; Hinds et al., 1987. *Mol. Cell. Biol. 7*:2863-2869; Finlay et al., 1988, *Mol. Cell. Biol. 8*:531-539; Sarnow et al., 1982, *Cell. 28*:387-394; Gronostajski et al., 1984, *Mol. Cell. Biol. 4*:442-448; Pinhasi-Kimhi et al., 1986, *Nature (Lond.) 320*:182-185; Ruscetti and Scolnick, 1983, *J. Virol. 46*:1022-1026; Pinhasi and Oren, 1984, *Mol. Cell. Biol. 4*:2180-2186; and Sturzbecher et al., 1987, *Oncogene 1*:201-211.) For example, p53 protein has been observed to form oligomeric protein complexes with the SV40 large T antigen, the adenovirus type 5 E1B-M, 55,000 protein, and the human papilloma virus type 16 or 18 E6 product. Linzer and Levine, 1979, *Cell 17*:43-52; Lane and Crawford, 1979, *Nature, 278*:261-263; Sarnow et al., 1982, *Cell 28*:387-394; Werness et al., 1990, *Science, 248*:76-79. Similarly, complexes have been observed of $p105^{RB}$ (the product of the retinoblastoma susceptibility gene) with T antigen (DeCaprio et al., 1988, *Cell 54*:275-283), the adenovirus EIA protein (Whyte et al., 1988, *Nature 334*:124-129) and the E7 protein of human papilloma virus 16 or 18 (Münger et al., 1989, *EMBO J. 8*:4099-4105). It has been suggested that interactions between these viral proteins and $p105^{RB}$ inactivate a growth-suppressive function of $p105^{RB}$, mimicking deletions and mutations commonly found in the RB gene in tumor cells. In a similar fashion, oligomeric protein complex

formation between these viral proteins and p53 may eliminate or alter the function of p53. Finlay et al., 1989, *Cell 57*:1083-1093.

Fakharzadeh et al. (*EMBO J. 10*:1565-1569, 1991) analyzed amplified DNA sequences present in a tumorigenic mouse cell line (*i.e.*, 3T3DM, a spontaneously transformed derivative of mouse Balb/c cells). Studies were conducted to determine whether any of the amplified genes induced tumorigenicity following introduction of the amplified genes into a nontransformed recipient cell (*e.g.*, mouse NIH3T3 or Rat2 cells). The resulting cell lines were tested for tumorigenicity in nude mice. A gene, designated MDM2, which is amplified more than 50-fold in 3T3DM cells, induced tumorigenicity when overexpressed in NIH3T3 and Rat 2 cells. From the nucleotide and predicted amino acid sequence of mouse MDM2 (mMDM2), Fakharzadeh speculated that this gene encodes a potential DNA binding protein that functions in the modulation of expression of other genes and, when present in excess, interferes with normal constraints on cell growth.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for diagnosing a neoplastic tissue, such as sarcoma, in a human.

It is another object of the invention to provide a cDNA molecule encoding the sequence of human MDM2.

Yet another object of the invention is to provide a preparation of human MDM2 protein which is substantially free of other human cellular proteins.

Still another object of the invention is to provide DNA probes capable of hybridizing with human MDM2 genes or mRNA molecules.

Another object of the invention is to provide antibodies immunoreactive with human MDM2 protein.

Still another object of the invention is to provide kits for detecting amplification or elevated expression of human MDM2.

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method of treating a neoplastic human cell.

Yet another object of the invention is to provide methods for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification.

Still another object of the invention is to provide polypeptides which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method for growing host cells containing a p53 expression vector.

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to human p53 *in vitro*. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C shows the cDNA sequence of human MDM2. In this figure, human and mouse nucleotide and amino acid sequences are compared, the mouse sequence being shown only where it differs from the corresponding human sequence.

Figure 2 shows that hMDM2 binds to p53.

Figure 3 illustrates the amplification of the hMDM2 gene in sarcomas.

Figure 4A-C illustrates hMDM2 expression.

Figure 5 shows the inhibition of p53-mediated transactivation by MDM2. Yeast were stably transfected with expression plasmids encoding p53, lex-VP16, MDM2 or the appropriate vector-only controls, as indicated. p53-responsive (bars a-c) or lexA-responsive (bars d-f) β-galactosidase reporter plasmids were used to assess the response.

Inset: Western blot analysis demonstrating MDM2 (90 kD) and p53 (53 kD) expression in representative yeast strains. The strain indicated by a plus was transfected with expression vector encoding full length MDM2 and p53, while the strain indicated by a minus was transfected only with the p53 expression vector.

Figure 6 shows the determination of MDM2 and p53 domains of interaction. Fig. 5A and Fig. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive β-galactosidase reporter). Yeast clones expressing β-galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined. β-galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. Fig. 6C. Random fragments of p53 were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Figure 7 shows protein expression from the yeast strains described in Figure 6. Western blot analysis was performed as described (Oliner, J.D., et al., *Nature 358*:80-83 (1992)), using 20 μg of protein per lane. The MDM2 and p53 codons contained in the fusion vectors are shown at the top of A and B, respectively. Fig. 7A. Upper panel probed with p53 Ab2 detecting p53; lower panel probed with anti-lexA polyclonal antibodies (lex Ab) detecting MDM2 fusion proteins of 30-50 kD. Fig. 7B. Upper panel probed with Lex Ab detecting the lexA-full length MDM2 fusion protein of 112 kD; lower panel probed with HA Ab (a monoclonal antibody directed against the hemagglutinin epitope tag, Berkeley Antibody) detecting p53 fusion proteins of approximately 25-30 kD.

Figure 8 shows the inhibition of the p53 activation domain by MDM2. Yeast were transfected with expression vectors encoding a lexA-p53 (p53 codons 1-73) fusion (bars a and b) or lexA alone (bar c). Strain b also expressed full length MDM2, and all strains contained the lexA-responsive β-galactosidase reporter plasmid. Inset: Upper panel probed with MDM2 polyclonal antibodies detecting full length MDM2 (90 kD); lower panel probed with lex Ab detecting the lex-p53 fusion protein of 40 kD.

Figure 9 shows a Western blot analysis using monoclonal antibodies to MDM2 or p53. Fifty μg of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis. The position of molecular weight markers, in kd, is given on the right.

Figure 10 demonstrates immunocytochemical analysis of OsA-CL and SW480 cells grown *in vitro*. Monoclonal antibody IF-2, specific for MDM2, and mAb 1801, specific for p53, were used. The exclusively nuclear localization of both proteins is evident, as is the higher expression of MDM2 protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for p53.

Figure 11 demonstrates MDM2 expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for MDM2. Tumors #3 and #10 showed nuclear expression of MDM2, while tumor #2 showed no staining.

DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that a gene exists which is amplified in some human tumors. The amplification of this gene, designated MDM2, is diagnostic of neoplasia or the potential therefor. Detecting the elevated expression of human MDM2-encoded products is also diagnostic of neoplasia or the potential for neoplastic transformation. Over a third of the sarcomas surveyed, including the most common bone and soft tissue forms, were found to have amplified hMDM2 sequences. Expression of hMDM2 was found to be correspondingly elevated in tumors with the gene amplification.

Other genetic alterations leading to elevated hMDM2 expression may be involved in tumorigenesis also, such as mutations in regulatory regions of the gene. Elevated expression of hMDM2 may also be involved in tumors other than sarcomas including but not limited to those in which p53 inactivation has been implicated. These include colorectal carcinoma, lung cancer and chronic myelogenous leukemia.

According to one embodiment of the invention, a method of diagnosing a neoplastic tissue in a human is provided. Tissue or body fluid is isolated from a human, and the copy number of human MDM2 genes is determined. Alternatively, expression levels of human MDM2 gene products can be determined. These include protein and mRNA.

Body fluids which may be tested include urine, serum, blood, feces, saliva, and the like. Tissues suspected of being neoplastic are desirably separated from normal appearing tissue for analysis. This can be done by paraffin or cryostat sectioning or flow cytometry, as is known in the art. Failure to separate neoplastic from non-neoplastic cells can confound the analysis. Adjacent non-neoplastic tissue or any normal tissue can be used to determine a base-line level of expression or copy number, against which the amount of hMDM2 gene or gene products can be compared.

The human MDM2 gene is considered to be amplified if the cell contains more than the normal copy number (2) of this gene per genome. The various techniques for detecting gene amplification are well known in the art. Gene amplification can be determined, for example, by Southern blot analysis, as described in Example 4, wherein cellular DNA from a human tissue is digested, separated, and transferred to a filter where it is hybridized with a probe containing complementary nucleic acids. Alternatively, quantitative polymerase chain reaction (PCR) employing primers can be used to determine gene amplification. Appropriate primers will bind to sequences that bracket human MDM2 coding sequences. Other techniques for determining gene copy number as are known in the art can be used without limitation.

The gene product which is measured may be either mRNA or protein. The term elevated expression means an increase in mRNA production or protein production over that which is normally produced by non-cancerous cells. Although amplification has been observed in human sarcomas, other genetic alterations leading to elevated expression of MDM2 may be present in these or other tumors. Other tumors include those of lung, breast, brain, colorectal, bladder, prostate, liver, skin, and stomach. These, too, are contemplated by the present invention. Non-cancerous cells for use in determining base-line expression levels can be obtained from cells surrounding a tumor, from other humans or from human cell lines. Any increase can have diagnostic value, but generally the mRNA or protein expression will be elevated at least about 3-fold, 5-fold, and in some cases up to about 100-fold over that found in non-cancerous cells. The particular technique employed for detecting mRNA or protein is not critical to the practice of the invention. Increased production of mRNA or protein may be detected, for example, using the techniques of Northern blot analysis or Western blot analysis, respectively, as described in Example 4 or other known techniques such as ELISA, immunoprecipitation, RIA and the like. These techniques are also well known to the skilled artisan.

According to another embodiment of the invention, nucleic acid probes or primers for the determining of human MDM2 gene amplification or elevated expression of mRNA are provided. The probe may comprise ribo- or deoxyribonucleic acids and may contain the entire human MDM2 coding sequence, a sequence complementary thereto, or fragments thereof. A probe may contain, for example, nucleotides 1-949, or 1-2372 as shown in Figure 1. Generally, probes or primers will contain at least about 14 contiguous nucleotides of the human sequence but may desirably contain about 40, 50 or 100 nucleotides. Probes are typically labelled with a fluorescent tag, a radioisotope, or the like to render them easily detectable. Preferably the probes will hybridize under stringent hybridization conditions. Under such conditions they will not hybridize to mouse MDM2. The probes of the invention are complementary to the human MDM2 gene. This means that they share 100% identity with the human sequence.

hMDM2 protein can be produced, according to the invention, substantially free of other human proteins. Provided with the DNA sequence, those of skill in the art can express the cDNA in a non-human cell. Lysates of such cells provide proteins substantially free of other human proteins. The lysates can be further purified, for example, by immunoprecipitation, co-precipitation with p53, or by affinity chromatography.

The antibodies of the invention are specifically reactive with hMDM2 protein. Preferably, they do not cross-react with MDM2 from other species. They can be polyclonal or monoclonal, and can be raised against native hMDM2 or a hMDM2 fusion protein or synthetic peptide. The antibodies are specifically immunoreactive with hMDM2 epitopes which are not present on other human proteins. Some antibodies are reactive with epitopes unique to human MDM2 and not present on the mouse homolog. The antibodies are useful in conventional analyses, such as Western blot analysis, ELISA, immunohistochemistry, and other immunological assays for the detection of proteins. Techniques for raising and purifying polyclonal antibodies are well known in the art, as are techniques for preparing monoclonal antibodies. Antibody binding can be determined by methods known in the art, such as use of an enzyme-labelled secondary antibody, staphylococcal protein A, and the like. Certain monoclonal antibodies of the invention have been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. These include IF2, and ED9, which have been granted accession nos. HB 11290, and HB 11291, respectively.

According to another embodiment of the invention, interference with the expression of MDM2 provides a therapeutic modality. The method can be applied in vivo, in vitro, or ex vivo. For example, expression may be down-regulated by administering triple-strand forming or antisense oligonucleotides which bind to the hMDM2 gene or mRNA, respectively, and prevent transcription or translation. The oligonucleotides may interact with unprocessed pre-mRNA or processed mRNA. Small molecules and peptides which specifically inhibit MDM2 expression can also be used.

Similarly, such molecules which inhibit the binding of MDM2 to p53 would be therapeutic by alleviating the sequestration of p53.

Such inhibitory molecules can be identified by screening for interference of the hMDM2/p53 interaction where one of the binding partners is bound to a solid support and the other partner is labeled. Antibodies specific for epitopes on hMDM2 or p53 which are involved in the binding interaction will interfere with such binding. Solid supports which may be used include any polymers which are known to bind proteins. The support may be in the form of a filter, column packing matrix, beads, and the like. Labeling of proteins can be accomplished according to any technique known in the art. Radiolabels, enzymatic labels, and fluorescent labels can be used advantageously. Alternatively, both hMDM2 and p53 may be in solution and bound molecules separated from unbound subsequently. Any separation technique known in the art may be employed, including immunoprecipitation or immunoaffinity separation with an antibody specific for the unlabeled binding partner.

It has been found that amino acid residues 13-41 of p53 (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and p53. However, additional residues on either the amino or carboxy terminal side of the peptide appear also to be required. Nine to 13 additional p53 residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from p53-regulated growth control in sarcomas, the use of p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53-regulated growth control.

Suitable p53-derived peptides for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

DNA encoding the MDM2-binding, p53-derived peptide, or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus,

or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, *inter alia* encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of peptide, the goal is to alleviate the sequestration of p53 by MDM2.

A cDNA molecule containing the coding sequence of hMDM2 can be used to produce probes and primers. In addition, it can be expressed in cultured cells, such as *E. coli*, to yield preparations of hMDM2 protein substantially free of other human proteins. The proteins produced can be purified, for example, with immunoaffinity techniques using the antibodies described above.

Kits are provided which contain the necessary reagents for determining gene copy number, such as probes or primers specific for the hMDM2 gene, as well as written instructions. The instructions can provide calibration curves to compare with the determined values. Kits are also provided to determine elevated expression of mRNA (*i.e.*, containing probes) or hMDM2 protein (*i.e.*, containing antibodies). Instructions will allow the tester to determine whether the expression levels are elevated. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to human p53. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

The following examples are provided to exemplify various aspects of the invention and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

To obtain human cDNA clones, a cDNA library was screened with a murine MDM2 (mMDM2) cDNA probe. A cDNA library was prepared by using polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 as a template for the production of random hexamer primed double stranded cDNA. Gubler and Hoffmann, 1983, *Gene 25*:263-268. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged, and plated as described by Elledge et al. (*Proc. Natl. Acad. Sci. USA, 88*:1731-1735, 1991). The library was screened initially with a P-labelled (Kinzler, K.W., et al., *Nucl. Acids Res. 17*:3645-3653 (1989), Feinberg and Vogelstein, 1983, *Anal. Biochem. 132*.6-13) mMDM2 cDNA probe (nucleotides 259 to 1508 (Fakharzadeh et al., 1991, *EMBO J. 10*:1565-1569)) and then rescreened with an hMDM2 cDNA clone containing nucleotides 40 to 702.

Twelve clones were obtained, and one of the clones was used to obtain thirteen additional clones by re-screening the same library. In total, twenty-five clones were obtained, partially or totally sequenced, and mapped. Sequence analysis of the twenty-five clones revealed several cDNA forms indicative of alternative splicing. The sequence shown in Figure 1 is representative of the most abundant class and was assembled from three clones: c14-2 (nucleotides 1-949), c89 (nucleotides 467-1737), and c33 (nucleotides 390-2372). The 3' end of the untranslated region has not yet been cloned in mouse or human. The 5' end is likely to be at or near nucleotide 1. There was an open reading frame extending from the 5' end of the human cDNA sequence to nucleotide 1784. Although the signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between hMDM2 and mMDM2 fell off dramatically upstream of nucleotide 312. This lack of conservation in an otherwise highly conserved protein suggested that the sequences upstream of the divergence may not code for protein. Second, an anchored polymerase chain reaction (PCR) approach was employed in an

effort to acquire additional upstream cDNA sequence. Ochman et al., 1985, In: *PCR Technology: Principles and Applications for DNA Amplification* (Erlich, ed.) pp. 105-111 (Stockton, New York). The 5' ends of the PCR derived clones were very similar (within 3 bp) to the 5' ends of clones obtained from the cDNA library, suggesting that the 5' end of the hMDM2 sequence shown in Figure 1 may represent the 5' end of the transcript. Third, *in vitro* translation of the sequence shown in Figure 1, beginning with the methionine encoded by the nucleotide 312 ATG, generated a protein similar in size to that observed in human cells.

In Figure 1, hMDM2 cDNA sequence, hMDM2 and mMDM2 nucleotide and amino acid sequences are compared. The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5' and 3' boundaries of the previously published mMDM2 cDNA. Fakharzadeh et al., 1991, *EMBO J. 10*:1565-1569. Dashes indicate insertions. The mouse and human amino acid sequences are compared from the putative translation start site at nucleotide 312 through the conserved stop codon at nucleotide 1784.

Comparison of the human and mouse MDM2 coding regions revealed significant conservation at the nucleotide (80.3%) and amino acid (80.4%) levels. Although hMDM2 and mMDM2 bore little similarity to other genes recorded in current databases, the two proteins shared several motifs. These included a basic nuclear localization signal (Tanaka, 1990, *FEBS Letters 271*:41-46) at codons 181 to 185, several casein kinase II serine phosphorylation sites (Pinna, 1990, *Biochem. et. Biophys. Acta. 1054*:267-284) at codons 166 to 169, 192 to 195, 269 to 272, and 290 to 293, an acidic activation domain (Ptashne, 1988, *Nature 355*:683-689) at codons 223 to 274, and two metal binding sites (Harrison, 1991, *Nature 353*:715) at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA binding domains. The protein kinase A domain noted in mMDM2 (Fakharzadeh et al., 1991, *EMBO J. 10*:1565-1569) was not conserved in hMDM2.

Example 2

To determine whether the hMDM2 protein could bind to human p53 protein *in vitro*, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Figure 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, *Nucleic Acid Research 15*:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, *Nature Genetics*, in press) and MCC (Kinzler et al., 1991, *Science 251*:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Although the predicted size of the protein generated from the construct was only 55.2 kd (extending from the methionine at nucleotide 312 to nucleotide 1784), *in vitro* translated protein migrated at approximately 95 kilodaltons.

Ten μl of lysate containing the three proteins (hMDM2, p53 and MCC), alone or mixed in pairs, were incubated at 37°C for 15 minutes. One microgram (10 μl) of p53 Ab1 (monoclonal antibody specific for the C-terminus of p53) or Ab2 (monoclonal antibody specific for the N-terminus of p53) (Oncogene Science), or 5 μl of rabbit serum containing MDM2 Ab (polyclonal rabbit anti-hMDM2 antibodies) or preimmune rabbit serum (obtained from the rabbit which produced the hMDM2 Ab), were added as indicated. The polyclonal rabbit antibodies were raised against an *E. coli*-produced hMDM2-glutathione S-transferase fusion protein containing nucleotides 390 to 816 of the hMDM2 cDNA. Ninety μl of RIPA buffer (10 mM tris [pH 7.5], 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer, or Binding Buffer (El-Deriy et al., 1992, *Nature Genetics*, in press) were then added and the mixtures allowed to incubate at 4°C for 2 hours.

Two milligrams of protein A sepharose were added to each tube, and the tubes were rotated end-over-end at 4°C for 1 hour. After pelleting and washing, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and the dried gels autoradiographed for 10 to 60 minutes in the presence of Enhance (New England Nuclear).

Figure 2 shows the co-precipitation of hMDM2 and p53. The three buffers produced similar results, although the co-precipitation was less efficient in SNNTE buffer containing 0.5 M NaCl (Figure 2, lanes 5 and 8) than in Binding Buffer containing 0.1 M NaCl (Figure 2 lanes 6 and 9).

In vitro translated hMDM2, p53 and MCC proteins were mixed as indicated above and incubated with p53 Ab1, p53 Ab2, hMDM2 Ab, or preimmune serum. Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. The bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (Figure 2, lanes 2 and 3). However, when *in vitro* translated human p53 was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association *in vitro* (Figure 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, *Science 251*:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (Figure 2, lanes 8 and 9). When an *in vitro* translated mutant form of p53 (175^{his}) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

In the converse of the experiments described above, the anti-hMDM2 antibodies immunoprecipitated p53 when mixed with hMDM2 protein (Figure 2, lane 15) but failed to precipitate p53 alone (Figure 5, lane 13). Preimmune rabbit serum failed to precipitate either hMDM2 or p53 (Figure 2, lane 16).

Example 3

In order to ascertain the chromosomal localization of hMDM2, somatic cell hybrids were screened with an hMDM2 cDNA probe. A human-hamster hybrid containing only human chromosome 12 was found to hybridize to the probe. Screening of hybrids containing portions of chromosome 12 (Turc-Carel et al., 1986, *Cancer Genet. Cytogenet. 23*:291-299) with the same probe narrowed the localization to chromosome 12q12-14.

Example 4

Previous studies have shown that this region of chromosome 12 is often aberrant in human sarcomas. Mandahl et al., 1987, Genes Chromosomes & Cancer 1:9-14; Turc-Carel et al., 1986, *Cancer Genet. Cytogenet. 23*:291-299; Meltzer et al., 1991, *Cell Growth & Differenriation 2*:495-501. To evaluate the possibility that hMDM2 was genetically altered in such cancers, Southern blot analysis was performed.

Figure 3 shows examples of the amplification of the hMDM2 gene in sarcomas. Cellular DNA (5 μg) was digested with *Eco*RI, separated by agarose gel electrophoresis, and transferred to nylon as described by Reed and Mann (*Nucl. Acids Res., 1985, 13*:7207-7215). The cellular DNA was derived from five primary sarcomas (lanes 1-4, 6) and one sarcoma cell line (OsA-C1, lane 5). The filters were then hybridized with an hMDM2 cDNA fragment probe nucleotide 1-949 (see Figure 1), or to a control probe which identifies fragments of similar size (DCC gene, 1.65 cDNA fragment). Fearon, 1989, *Science 247*:49-56. Hybridization was performed as described by Vogelstein et al. (*Cancer Research, 1987, 47*:4806-4813). A striking amplification of hMDM2 sequences was observed in several of these tumors. (See Figure 3, lanes 2, 3 and 5). Of 47 sarcomas analyzed, 17 exhibited hMDM2 amplification ranging from 5 to 50 fold. These tumors included 7 to 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas (MFH), 3 of 11 osteosarcomas, and 0 and 1 rhabdomyosarcomas. Five benign soft tissue tumors (lipomas) and twenty-seven carcinomas (colorectal or gastric) were also tested by Southern blot analysis and no amplification was observed.

Example 5

This example illustrates that gene amplification is associated with increased expression.

Figure 4A illustrates hMDM2 expression as demonstrated by Northern blot analysis. Because of RNA degradation in the primary sarcomas, only the cell lines could be productively analyzed by Northern blot. RNA was separated by electrophoresis in a MOPS-formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were performed as described by Kinzler et al. (*Nature 332*:371-374, 1988). The RNA was hybridized to the hMDM2 fragment described in Figure 3. Ten μg of total RNA derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains 10 μg of polyadenylated CaCo-2 RNA. RNA sizes are shown in kb. In the one available sarcoma cell line with hMDM2 amplification, a single transcript of approximately 5.5 kb was observed (Figure 4A, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Figure 4A, lane 2) or in a carcinoma cell line (Figure 4A, lane 3). When purified mRNA (rather than total RNA) from the carcinoma cell line was used for analysis, an hMDM2 transcript of 5.5 kb could also be observed (Figure 4A, lane 4).

Figure 4B illustrates hMDM2 expression as demonstrated by Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2).

Figure 4C illustrates hMDM2 expression as demonstrated by Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with hMDM2 amplifications, and lanes 4 and 5 contain protein from sarcomas without hMDM2 amplification.

Western blots using affinity purified MDM2 Ab were performed with 50 μg protein per lane as described by Kinzler et al. (*Mol. Cell. Biol., 1990, 10*:634-642), except that the membranes were blocked in 10% nonfat dried milk and 10% goat serum,

and secondary antibodies were coupled to horseradish peroxidase, permitting chemiluminescent detection (Amersham ECL). MDM2 Ab was affinity purified with a pATH-hMDM2 fusion protein using methods described in Kinzler et al. (*Mol. Cell. Biol. 10*:634-642, 1990). Non-specifically reactive proteins of about 75-85, 105-120 and 170-200 kd were observed in all lanes, irrespective of hMDM2 amplification status. hMDM2 proteins, of about 90-97 kd, were observed only in the hMDM2-amplified tumors. Protein marker sizes are shown in kd.

A protein of approximately 97 kilodaltons was expressed at high levels in the sarcoma cell line with hMDM2 amplification (Figure 4B, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Figure 4B, lanes 1, 2 and 4). Five primary sarcomas were also examined by Western blot analysis. Three primary sarcomas with amplification expressed the same size protein as that observed in the sarcoma cell line (Figure 4C, lanes 1-3), while no protein was observed in the two sarcomas without amplification (Figure 4C, lanes 4 and 5).

Expression of the hMDM2 RNA in the sarcoma with amplification was estimated to be at least 30 fold higher than that in the other lines examined. This was consistent with the results of Western blot analysis.

The above examples demonstrate that hMDM2 binds to p53 *in vitro* and is genetically altered (*i.e.*, amplified) in a significant fraction of sarcomas, including MFH, liposarcomas, and osteosarcomas. These are the most common sarcomas of soft tissue and bone. Weiss and Enzinger, 1978, *Cancer 41*:2250-2266; Malawer et al., 1985, In: *Cancer: Principles and Practice of Oncology*, DeVita et al., Eds., pp. 1293-1342 (Lippincott, Philadelphia).

Human MDM2 amplification is useful for understanding the pathogenesis of these often lethal cancers.

MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as SV40 T-antigen, adenovirus E1B, and HPV E6. Lane and Bechimol, 1990. *Genes and Development 4*:1-8; Werness et al., 1990, *Science*

248:76. Consistent with this hypothesis, no sarcomas with hMDM2 amplification had any of the p53 gene mutations that occur commonly in other tumors. hMDM2 amplification provides a parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. The finding that expression of hMDM2 is correspondingly elevated in tumors with amplification of the gene are consistent with the finding that MDM2 binds to p53, and with the hypothesis that overexpression of MDM2 in sarcomas allows escape from p53 regulated growth control. This mechanism of tumorigenesis has striking parallels to that previously observed for virally induced tumors (Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, *Science 248*:76), in which viral oncogene products bind to and functionally inactivate p53.

Example 6

This example demonstrates that MDM2 expression inhibits p53-mediated transactivation.

To determine if MDM2 could influence the ability of p53 to activate transcription, expression vectors coding for the two proteins were stably transfected into yeast along with a p53-responsive reporter construct. The reporter consisted of a β-galactosidase gene under the transcriptional control of a minimal promoter and a multimerized human DNA sequence which strongly bound p53 *in vitro* (Kern, S.E., et al., *Science 256*:827-830 (1992). Reporter expression was completely dependent on p53 in this assay (Figure 5, compare bars a and c). MDM2 expression was found to inhibit p53-mediated transactivation of this reporter 16-fold relative to isogeneic yeast lacking MDM2 expression (Figure 5, compare bars a and b). Western blot analysis confirmed that p53 (53 kD) was expressed equivalently in strains with and without MDM2 (90 kD) (Figure 1, inset).

METHODS. The MDM2 expression plasmid, pPGK-MDM2, was constructed by inserting the full length MDM2 cDNA (Oliner, J.D., et al., *Nature 358*:80-83 (1992)) into pPGK (Poon, D. *et al., Mol. and Cell.*

Biol. 1111:4809-4821 (1991)), immediately downstream of the phosphoglycerate kinase constitutive promoter. Galactose-inducible p53 (pRS314SN, Nigro, J.M., et al., *Mol. and Cell. Biol. 12*:1357-1365 (1992)), lexA-VP16 (YVLexA, Dalton, S., et al., *Cell 68*:597-612 (1992)), and lexA (YLexA, YVLexA minus VP16) plasmids were used as indicated. The reporters were PG16-*lacZ* (Kern, S.E. *et al., Science 256*:827-830 (1992)) (p53-responsive) and pJK103 (Kamens, J., et al., *Mol. Cell. Biol. 10*:2840-2847 (1990)) (lexA-responsive). *S. cerevisiae* strain pEGY48 was transformed as described (Kinzler, K.W. et al., *Nucl. Acids Res. 17*:3645-3653 (1989)). Yeast strains represented by bars a-c were grown at 30°C to mid-log phase in selective liquid medium containing 2% raffinose as the carbon source, induced for 30 minutes by the addition of 2% galactose, harvested, and lysed as described (Kern, S.E. *et al., Science 256*:827-830 (1992)). The strains represented by bars d-f were treated similarly, except that the cells were induced in galactose for 4 hours to obtain measurable levels of β-galactosidase. β-galactosidase activities shown represent the mean of three to five experimental values (error bars indicate s.e.m.). Protein concentrations were determined by a Coomassie blue-based (bio-Rad) assay. The β-galactosidase assays were performed with AMPGD chemiluminescent substrate and Emerald enhancer (Tropix) according to the manufacturer's instructions. β-galactosidase activities of bars b and c are shown relative to that of bar A; β-galactosidase activities of bars e and f are shown relative to that of bar d. Western blots were performed as described (Oliner, J.D., et al., *Nature 358*:80-83 (1992)), using p53 Ab1801 (lower panel, Oncogene Science) or MDM2 polyclonal antibodies (Oliner, J.D., et al., *Nature 358*:80-83 (1992)) (upper panel).

To ensure that this inhibition was not simply a general transcriptional down regulation mediated by the expression of the foreign MDM2 gene, a yeast strain was created that contained a different transcriptional activator (lexA-VP16, consisting of the lexA DNA binding domain fused to the VP16 acidic activation domain), a similar reporter (with a lexA-responsive site upstream of a β-galactosidase gene), and the same MDM2 expression vector. The results shown in Figure 1 (bars d & e) demonstrate that lexA-VP16 transactivation was unaffected by the presence of MDM2. Furthermore, MDM2 expression had no apparent effect on the growth rate of the cells.

Example 7

This example demonstrates the domains of p53 and MDM2 which interact with each other.

To gain insight into the mechanism of the MDM2-mediated p53 inhibition, the domains of MDM2 and p53 responsible for binding to one another were mapped. The yeast system used to detect protein-protein binding takes advantage of the modular nature of transcription factor domains (Keegan, L., et al., *Science 231*:699-704 (1986); Chien, C.-T., *Proc. Natl. Acad. Sci. U.S.A. 88*:9578-9582 (1991); Brent, R., et al., *Cell 43*:729-731 (1985); Ma, J., et al., *Cell 55*:4430446 (1988). Generically, if protein 1 (fused to a sequence-specific DNA binding domain) is capable of binding to protein 2 (fused to a transcriptional activation domain), then co-expression of both fusion proteins will result in transcriptional activation of a suitable reporter. In our experiments, the lexA DNA binding domain (amino acids 2-202) and the B42 acidic activation domain (AAD) were used in the fusion constructs. The reporter (Kamens, J., et al., *Mol. Cell. Biol. 10*:2840-2847 (1990); contained a lexA-responsive site upstream of a β-galactosidase gene. As an initial control experiment, full length MDM2 was inserted into the lexA fusion vector, and full length p53, supplying its intrinsic activation domain was inserted into a non-fusion vector. The combination resulted in the activation of the lexA-responsive reporter, while the same expression constructs lacking either the MDM2 or p53 cDNA inserts failed to activate β-galactosidase (Table I, strains 1, 2, and 3). Thus, activation was dependent upon MDM2-p53 binding.

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA fragments encoding MDM2 or p53 were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K.W., et al., *Nucl. Acids Res. 17*:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D.M., et al., *Nature 350*:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive β-galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent clones are diagrammed in Fig. 6A. The MDM2 sequences of the remaining 15 p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of Fig. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-terminal to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three p53 sequences shown in Fig. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated fragments.

The resultant yeast colonies were examined for β-galactosidase activity *in situ*. Of approximately 5000 clones containing MDM2 fragments fused to the lexA DNA

binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of β-galactosidase (about 5-fold less than the other fifteen clones) and β-galactosidase expression was independent of p53 expression (Figure 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable β-galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino terminal region of MDM2 (Figure 6B). The β-galactosidase activity of these clones was dependent on p53 co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length p53 contained MDM2 codons 1 to 118 (Figure 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated fragments of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

In a converse set of experiments, yeast clones containing fragments of p53 fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (Figure 2C). The minimal overlap between these three fragments contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the fragments required 9-12 amino acids on either side of codons 13-41 for activity (Figure 6C). To further test the idea that the amino terminal region of p53 was required for MDM2 binding, we generated an additional yeast strain expressing

the lexA-DNA binding domain fused to p53 codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-terminus of p53 were required for the interaction.

TABLE I

STRAIN NUMBER	p53 CONSTRUCT	MDM2 CONSTRUCT	ACTIVATION
1	p53[a]	Vector[b]	-
2	p53[a]	lexA-MDM2[b]	+
3	Vector[a]	lexA-MDM2[b]	-
4	p53[a]	lexA-MDM2 (1-118)[b]	+
5	Vector[a]	lexA-MDM2 (1-118)[b]	-
6	B42-p53 (1-41)[c]	lexA-MDM2[b]	+
7	B42-p53 (1-41)[c]	Vector[b]	-
8	lexA-p53 (74-393)[b]	B42-MDM2[c]	-
9	p53 (1-137)[a]	lexA-MDM2[b]	-

The MDM2 and p53 proteins expressed in each strain, along with the relevant reporters, are indicated. Numbers in parentheses refer to the MDM2 or p53 amino acids encoded (absence of parentheses indicated full length protein, that is, MDM2 amino acids 1 to 491 or p53 amino acids 1 to 393). The lexA-responsive β-galactosidase reporter plasmid (pJK103, Kamens, J., et al., *Mol. Cell. Biol. 10*:2840-2847 (1990)) was present in all strains.

[a]pRS314 vector (Nigro, J.M., et al., *Mol. and Cell. Biol. 12*:1357-1365 (1992).

[b]plex(1-202)+PL vector, containing lexA DNA binding domain fused to insert (Ruden, D.M., et al., *Nature 350*:250-252 (1991).

[c]pJG4-5 vector, containing B42 activation domain fused to insert.

[d](+) indicates that colonies turned blue following 24 hours of incubation on X-gal-containing selective medium, while (-) indicates that colonies remained white following 72 hours of incubation.

Sequence analysis showed that all p53 and MDM2 fragments noted in Figure 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in Figure 6 expressed the relevant proteins at similar levels, as shown by Western blotting (Figure 7).

The most striking results of these mapping experiments was that the region of p53 required to bind MDM2 was almost identical to the previously identified acidic activation domain of p53 (amino acids 20-42) (Unger, T., et al., *EMBO J. 11*:1383-1390 (1992); Miller, C.W., et al., *Proc. Am. Assoc. Cancer Res. 33*:386 (1992). This suggested that MDM2 inhibits p53-mediated transcriptional activation by "concealing" the activation domain of p53 from the transcriptional machinery. If this were true, the p53 activation domain, in isolation from the rest of the p53 protein, should still be inhibitable by full length MDM2. To test this hypothesis, we produced a hybrid protein containing the p53 activation domain (codons 1-73) fused to the lexA-DNA binding domain. This construct exhibited strong transcriptional activation of a lexA-responsive reporter (Figure 8), as predicted from previous experiments in which the p53 activation domain was fused to another DNA binding domain (Fields, S., et al., *Science 249*:1046-1049 (1990); Raycroft, L., et al., *Science 249*:1049-1051 (1990)). The lexA-p53 DNA construct was stably expressed in yeast along with the full length MDM2 expression vector (or the vector alone). MDM2 expression resulted in a five-fold decrease in reporter activity, demonstrating that MDM2 can specifically inhibit the function of the p53 activation domain regardless of the adjacent protein sequences tethering p53 to DNA (Figure 8).

METHODS. Strains were grown to mid-log phase in 2% dextrose before induction of p53 expression for 2 hours by the addition of 2% galactose. The lex-p53 construct was identical to lex-VP16 (YVlexA, Dalton, S., et al., *Cell 68*:597-612 (1992)) except that VP16 sequences were replaced by p53 sequences encoding amino acids 1 to 73.

The results obtained in the experiments discussed herein raise an interesting paradox. If MDM2 binds to (Figure 6) and conceals (Figure 8) the p53 activation

domain from the transcriptional machinery, how could the lexA-MDM2-p53 complex activate transcription from the lexA-responsive reporter (Table I, strain 2)? Because the only functional activation domain in the lexA-MDM2-p53 complex of strain 2 is expected to be contributed by p53, one might predict that it would be concealed by binding to MDM2 and thereby fail to activate. A potential resolution of this paradox is afforded by knowledge that p53 exists as a homotetramer (Stenger, J.E., et al., *Mol. Carcinogenesis* 5:102-106 (1992); Sturzbecher, H.W. *et al.*, *Oncogene* 7:1513-1523 (1992). Thus the activation noted in the lexA-MDM2-p53 complex could be due to the presence of four individual activation domains contributed by the p53 tetramer, not all of which were concealed by MDM2. As a direct test of this issue, the domain of p53 required for homo-oligomerization (Stenger, J.E., et al., *Mol. Carcinogenesis* 5:102-106 (1992); Sturzbecher, H.W. *et al.*, *Oncogene* 7:1513-1523 (1992) (the C-terminus) was removed from the p53 expression construct, so that it consisted of only codons 1-137. This truncated p53 polypeptide retained the entire activation domain (as shown in Figure 8, bar a) and the entire domain required for interaction with MDM2 (Table I, strain 6). Yet, when allowed to interact with lexA-MDM2, no transactivation of the lexA-responsive reporter was observed (Table I, strain 9). Because p53 did not inhibit lexA-MDM2 binding to the lexA reporter (Table I, strain 2), the result of strain 9 is likely to be due to a direct inhibition of the isolated p53 activation domain by MDM2.

Example 8

This example illustrates the production and characterization of antibodies specific for MDM2 epitopes.

The antigen preparations used to intraperitoneally immunize female (BALB/c X C57BL/6)F1 mice comprised bacterially expressed, glutathione-column purified glutathione-S-transferase-MDM2 (GST-MDM2) fusion protein. (One preparation was further purified on a polyacrylamide gel and electroeluted.) The fusion protein contains a 16 kD amino terminal portion of human MDM2 protein (amino acids 27 to

168). For immunization, the fusion protein was mixed with Ribi adjuvant (Ribi Immunochem Research, Inc.).

Two mice were sacrificed and their spleen cells fused to SP2/0s myeloma cells (McKenzie, et al., *Oncogene*, *4*:543-548, 1989). Resulting hybridomas were screened by ELISA on trpE-MDM2 fusion protein-coated microtiter wells. The trpE-MDM2 fusion protein contains the same portion of MDM2 as the GST-MDM2 fusion protein. Antigen was coated at a concentration of 1 μg/ml.

A second fusion was performed as described except hybridomas were screened on electroeluted, glutathione purified GST-MDM2. Positive hybridomas from both fusions were expanded and single cell subcloned. Subclones were tested by Western Blot for specificity to the 55 kD trpE-MDM2 and the 43 kD GST-MDM2 fusion proteins.

Two Western Blot positive subclones (1F2 and JG3) were put into mice for ascites generation. The resulting ascites were protein A purified. Both purified monoclonal antibodies tested positive by Western Blot and immunoprecipitation for the 90 kD migrating MDM2 protein present in a human osteosarcoma cell line (OsA-CL), which overexpresses MDM2, and negative in the HOS osteosarcoma, which does not overexpress MDM2.

ED9 was protein G-purified from ascites and found to be specific in cryostat immunohistochemistry for MDM2 in osteosarcoma cells, as was IF2.

Example 9

This example demonstrates the expression and detection of MDM2 at the cellular level.

To evaluate MDM2 expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. (See example 8.) Of several antibodies tested, mAb IF-2 was the most useful, as it detected MDM2 in several assays. For initial testing, we compared proteins derived

from OsA-CL, a sarcoma cell line with MDM2 amplification but without p53 mutation (Table II) and proteins from SW480, a colorectal cancer cell line with p53 mutation (Barak et al., *EMBO 12*:461-468 (1993)) but without MDM2 amplification (data not shown). Figure 9 shows that the mAb IF-2 detected an intense 90 kd band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense 90 kd band in SW480 extracts. We could not distinguish whether the low molecular weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in MDM2 gene copy number in these two lines. Conversely, the 53 kd signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL consistent with the presence of a mutated p53 in SW480 (Fig. 9).

Cells grown on cover slips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 10). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (Barak et al., *EMBO 12*:461-468 (1993)) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186. Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 10), with the relative intensities consistent with the Western blot results (Fig. 9).

The IF-2 mAb was then used (at 5 μg/ml) to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors #3 and #10) stained strongly (Fig. 11). Both of these tumors contained MDM2 gene amplification (Table II). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 11).

TABLE II

TUMOR #	TUMOR ID	TYPE[a]	MDM2 AMPLIFICATION[b]	P53 ALTERATION[c]	OVER-EXPRESSION[d]
1	M-2	MFH	ABSENT	DELETION/ REARRANGEMENT	NONE
2	M-5	MFH	ABSENT	CGC-CUC MUTATION; Arg(158)-His	p53
3	M-7	MFH	PRESENT	NONE OBSERVED	MDM2
4	M-8	MFH	ABSENT	DELETION	NONE
5	M-14	MFH	ABSENT	NONE OBSERVED	N.T.
6	M-15	MFH	ABSENT	DELETION	N.T.
7	M-16	MFH	ABSENT	NONE OBSERVED	NONE
8	M-17	MFH	ABSENT	NONE OBSERVED	N.T.
9	M-18	MFH	ABSENT	OVEREXPRESSED	p53
10	M-20	MFH	PRESENT	NONE OBSERVED	MDM2
11	L-5	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
12	L-7	LIPOSARCOMA	ABSENT	AAC-AGC MUTATION; Asn(239)-Ser	N.T.
13	L-9	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.

TABLE II (Cont.)

TUMOR #	TUMOR ID	TYPE[a]	MDM2 AMPLIFICATION[b]	P53 MUTATION[c]	OVER-EXPRESSION[d]
14	L-11	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
15	KL5B	LIPOSARCOMA	ABSENT	CAG-UAG MUTATION; Gln(144)-Stop	N.T.
16	KL7	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
17	KL10	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
18	KL11	LIPOSARCOMA	ABSENT	GGT-GAT MUTATION; EXON 5 SPLICE DONOR SITE	N.T.
19	KL12	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
20	KL28	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
21	KL30	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
22	S189	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
23	S131B	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
24	OSA-CL	MFH	PRESENT	NONE OBSERVED	MDM2

[a] MFH= malignant fibrous histiocytoma

[b] as assessed by Southern blot

[c] as assessed by Southern blot, sequencing of exons 5-8, or immunohistochemical analysis

[d] as assessed by immunohistochemical analysis; N.T. = not tested

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BURRELL, MARILEE
HILL, DAVID E.
KINZLER, KENNETH W.
VOGELSTEIN, BERT

(ii) TITLE OF INVENTION: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: BANNER, BIRCH, MCKIE AND BECKETT
(B) STREET: 1001 G STREET, N.W.
(C) CITY: WASHINGTON
(D) STATE: D.C.
(E) COUNTRY: USA
(F) ZIP: 20001

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 07-APR-1993

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: KAGAN, SARAH A.

(B) REGISTRATION NUMBER: 32,141

(C) REFERENCE/DOCKET NUMBER: 01107.42798

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 202-508-9100

(B) TELEFAX: 202-508-9299

(C) TELEX: 197430 BBMB UT

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 64 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 17q

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln
1 5 10 15

Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu
20 25 30

Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp
35 40 45

Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro
50 55 60

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2372 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(H) CELL LINE: CaCo-2

(viii) POSITION IN GENOME:

(B) MAP POSITION: 12q12-14

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 312..1784

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGGCC CTGTGTGTCG GAAAGATGGA 60

GCAAGAAGCC GAGCCCGAGG GGCGGCCGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG 120

CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG 180

GAGAGTGGAA TGATCCCCGA GGCCCAGGGC GTCGTGCTTC CGCAGTAGTC AGTCCCCGTG 240

AAGGAAACTG GGGAGTCTTG AGGGACCCCC GACTCCAAGC GCGAAAACCC CGGATGGTGA 300

GGAGCAGGCA A ATG TGC AAT ACC AAC ATG TCT GTA CCT ACT GAT GGT GCT 350
Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala
1 5 10

GTA ACC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACC CTG GTT AGA 398
Val Thr Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg
15 20 25

CCA AAG CCA TTG CTT TTG AAG TTA TTA AAG TCT GTT GGT GCA CAA AAA 446
Pro Lys Pro Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Lys
30 35 40 45

GAC ACT TAT ACT ATG AAA GAG GTT CTT TTT TAT CTT GGC CAG TAT ATT 494
Asp Thr Tyr Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile
50 55 60

ATG ACT AAA CGA TTA TAT GAT GAG AAG CAA CAA CAT ATT GTA TAT TGT 542
Met Thr Lys Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys
65 70 75

TCA AAT GAT CTT CTA GGA GAT TTG TTT GGC GTG CCA AGC TTC TCT GTG 590
Ser Asn Asp Leu Leu Gly Asp Leu Phe Gly Val Pro Ser Phe Ser Val
80 85 90

```
AAA GAG CAC AGG AAA ATA TAT ACC ATG ATC TAC AGG AAC TTG GTA GTA      638
Lys Glu His Arg Lys Ile Tyr Thr Met Ile Tyr Arg Asn Leu Val Val
 95                  100                 105

GTC AAT CAG CAG GAA TCA TCG GAC TCA GGT ACA TCT GTG AGT GAG AAC      686
Val Asn Gln Gln Glu Ser Ser Asp Ser Gly Thr Ser Val Ser Glu Asn
110                 115                 120                 125

AGG TGT CAC CTT GAA GGT GGG AGT GAT CAA AAG GAC CTT GTA CAA GAG      734
Arg Cys His Leu Glu Gly Gly Ser Asp Gln Lys Asp Leu Val Gln Glu
                130                 135                 140

CTT CAG GAA GAG AAA CCT TCA TCT TCA CAT TTG GTT TCT AGA CCA TCT      782
Leu Gln Glu Glu Lys Pro Ser Ser Ser His Leu Val Ser Arg Pro Ser
            145                 150                 155

ACC TCA TCT AGA AGG AGA GCA ATT AGT GAG ACA GAA GAA AAT TCA GAT      830
Thr Ser Ser Arg Arg Arg Ala Ile Ser Glu Thr Glu Glu Asn Ser Asp
        160                 165                 170

GAA TTA TCT GGT GAA CGA CAA AGA AAA CGC CAC AAA TCT GAT AGT ATT      878
Glu Leu Ser Gly Glu Arg Gln Arg Lys Arg His Lys Ser Asp Ser Ile
    175                 180                 185

TCC CTT TCC TTT GAT GAA AGC CTG GCT CTG TGT GTA ATA AGG GAG ATA      926
Ser Leu Ser Phe Asp Glu Ser Leu Ala Leu Cys Val Ile Arg Glu Ile
190                 195                 200                 205
```

TGT TGT GAA AGA AGC AGT AGC AGT GAA TCT ACA GGG ACG CCA TCG AAT 974
Cys Cys Glu Arg Ser Ser Ser Ser Glu Ser Thr Gly Thr Pro Ser Asn
210 215 220

CCG GAT CTT GAT GCT GGT GTA AGT GAA CAT TCA GGT GAT TGG TTG GAT 1022
Pro Asp Leu Asp Ala Gly Val Ser Glu His Ser Gly Asp Trp Leu Asp
225 230 235

CAG GAT TCA GTT TCA GAT CAG TTT AGT GTA GAA TTT GAA GTT GAA TCT 1070
Gln Asp Ser Val Ser Asp Gln Phe Ser Val Glu Phe Glu Val Glu Ser
240 245 250

CTC GAC TCA GAA GAT TAT AGC CTT AGT GAA GAA GGA CAA GAA CTC TCA 1118
Leu Asp Ser Glu Asp Tyr Ser Leu Ser Glu Glu Gly Gln Glu Leu Ser
255 260 265

GAT GAA GAT GAT GAG GTA TAT CAA GTT ACT GTG TAT CAG GCA GGG GAG 1166
Asp Glu Asp Asp Glu Val Tyr Gln Val Thr Val Tyr Gln Ala Gly Glu
270 275 280 285

AGT GAT ACA GAT TCA TTT GAA GAA GAT CCT GAA ATT TCC TTA GCT GAC 1214
Ser Asp Thr Asp Ser Phe Glu Glu Asp Pro Glu Ile Ser Leu Ala Asp
290 295 300

TAT TGG AAA TGC ACT TCA TGC AAT GAA ATG AAT CCC CCC CTT CCA TCA 1262
Tyr Trp Lys Cys Thr Ser Cys Asn Glu Met Asn Pro Pro Leu Pro Ser
305 310 315

```
CAT TGC AAC AGA TGT TGG GCC CTT CGT GAG AAT TGG CTT CCT GAA GAT      1310
His Cys Asn Arg Cys Trp Ala Leu Arg Glu Asn Trp Leu Pro Glu Asp
320                 325                 330

AAA GGG AAA GAT AAA GGG GAA ATC TCT GAG AAA GCC AAA CTG GAA AAC      1358
Lys Gly Lys Asp Lys Gly Glu Ile Ser Glu Lys Ala Lys Leu Glu Asn
335                 340                 345

TCA ACA CAA GCT GAA GAG GGC TTT GAT GTT CCT GAT TGT AAA AAA ACT      1406
Ser Thr Gln Ala Glu Glu Gly Phe Asp Val Pro Asp Cys Lys Lys Thr
350                 355                 360                 365

ATA GTG AAT GAT TCC AGA GAG TCA TGT GTT GAG GAA AAT GAT GAT AAA      1454
Ile Val Asn Asp Ser Arg Glu Ser Cys Val Glu Glu Asn Asp Asp Lys
370                 375                 380

ATT ACA CAA GCT TCA CAA TCA CAA GAA AGT GAA GAC TAT TCT CAG CCA      1502
Ile Thr Gln Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro
385                 390                 395

TCA ACT TCT AGT AGC ATT ATT TAT AGC AGC CAA GAA GAT GTG AAA GAG      1550
Ser Thr Ser Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu
400                 405                 410

TTT GAA AGG GAA GAA ACC CAA GAC AAA GAA GAG AGT GTG GAA TCT AGT      1598
Phe Glu Arg Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser
415                 420                 425
```

TTG CCC CTT AAT GCC ATT GAA CCT TGT GTG ATT TGT CAA GGT CGA CCT 1646
Leu Pro Leu Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro
430 435 440 445

AAA AAT GGT TGC ATT GTC CAT GGC AAA ACA GGA CAT CTT ATG GCC TGC 1694
Lys Asn Gly Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys
450 455 460

TTT ACA TGT GCA AAG AAG CTA AAG AAA AGG AAT AAG CCC TGC CCA GTA 1742
Phe Thr Cys Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val
465 470 475

TGT AGA CAA CCA ATT CAA ATG ATT GTG CTA ACT TAT TTC CCC 1784
Cys Arg Gln Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro
480 485 490

TAGTTGACCT GTCTATAAGA GAATTATATA TTTCTAACTA TATAACCCTA GGAATTTAGA 1844

CAACCTGAAA TTTATTCACA TATATCAAAG TGAGAAAATG CCTCAATTCA CATAGATTTC 1904

TTCTCTTTAG TATAATTGAC CTACTTTGGT AGTGGAATAG TGAATACTTA CTATAATTTG 1964

ACTTGAATAT GTAGCTCATC CTTTACACCA ACTCCTAATT TTAAATAATT TCTACTCTGT 2024

CTTAAATGAG AAGTACTTGG TTTTTTTTTT CTTAAATATG TATATGACAT TTAAATGTAA 2084

CTTATTATTT TTTTTGAGAC CGAGTCTTGC TCTGTTACCC AGGCTGGAGT GCAGTGGGTG 2144

ATCTTGGCTC ACTGCAAGCT CTGCCCTCCC CGGGTTCGCA CCATTCTCCT GCCTCAGCCT 2204

CCCAATTAGC TTGGCCTACA GTCATCTGCC ACCACACCTG GCTAATTTTT TGTACTTTTA 2264

GTAGAGACAG GGTTTCACCG TGTTAGCCAG GATGGTCTCG ATCTCCTGAC CTCGTGATCC 2324

GCCCACCTCG GCCTCCCAAA GTGCTGGGAT TACAGGCATG AGCCACCG 2372

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 491 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr
1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr
35 40 45

Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys
50 55 60

Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys Ser Asn Asp
65 70 75 80

Leu Leu Gly Asp Leu Phe Gly Val Pro Ser Phe Ser Val Lys Glu His
85 90 95

Arg Lys Ile Tyr Thr Met Ile Tyr Arg Asn Leu Val Val Val Asn Gln
100 105 110

Gln Glu Ser Ser Asp Ser Gly Thr Ser Val Ser Glu Asn Arg Cys His
115 120 125

Leu Glu Gly Gly Ser Asp Gln Lys Asp Leu Val Gln Glu Leu Gln Glu
130 135 140

Glu Lys Pro Ser Ser Ser His Leu Val Ser Arg Pro Ser Thr Ser Ser
145 150 155 160

Arg Arg Arg Ala Ile Ser Glu Thr Glu Glu Asn Ser Asp Glu Leu Ser
165 170 175

Gly Glu Arg Gln Arg Lys Arg His Lys Ser Asp Ser Ile Ser Leu Ser
180 185 190

Phe Asp Glu Ser Leu Ala Leu Cys Val Ile Arg Glu Ile Cys Cys Glu
195 200 205

Arg Ser Ser Ser Ser Glu Ser Thr Gly Thr Pro Ser Asn Pro Asp Leu
210 215 220

Asp Ala Gly Val Ser Glu His Ser Gly Asp Trp Leu Asp Gln Asp Ser
225 230 235 240

Val Ser Asp Gln Phe Ser Val Glu Phe Glu Val Glu Ser Leu Asp Ser
245 250 255

Glu Asp Tyr Ser Leu Ser Glu Glu Gly Gln Glu Leu Ser Asp Glu Asp
260 265 270

Asp Glu Val Tyr Gln Val Thr Val Tyr Gln Ala Gly Glu Ser Asp Thr
275 280 285

Asp Ser Phe Glu Glu Asp Pro Glu Ile Ser Leu Ala Asp Tyr Trp Lys
290 295 300

Cys Thr Ser Cys Asn Glu Met Asn Pro Pro Leu Pro Ser His Cys Asn
305 310 315 320

Arg Cys Trp Ala Leu Arg Glu Asn Trp Leu Pro Glu Asp Lys Gly Lys
325 330 335

Asp Lys Gly Glu Ile Ser Glu Lys Ala Lys Leu Glu Asn Ser Thr Gln
340 345 350

Ala Glu Glu Gly Phe Asp Val Pro Asp Cys Lys Lys Thr Ile Val Asn
355 360 365

Asp Ser Arg Glu Ser Cys Val Glu Glu Asn Asp Asp Lys Ile Thr Gln
370 375 380

Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro Ser Thr Ser
385 390 395 400

Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu Phe Glu Arg
405 410 415

Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser Leu Pro Leu
420 425 430

Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly
435 440 445

Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys Phe Thr Cys
450 455 460

Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln
465 470 475 480

Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro
485 490

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1710 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Mus musculus

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 202..1668

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT 60

CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC 120

TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGA CCCTCTCGGA TCACCGCGCT 180

TCTCCTGCGG CCTCCAGGCC A ATG TGC AAT ACC AAC ATG TCT GTG TCT ACC 231
Met Cys Asn Thr Asn Met Ser Val Ser Thr
1 5 10

GAG GGT GCT GCA AGC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACT 279
Glu Gly Ala Ala Ser Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr
15 20 25

```
CTG GTT AGA CCA AAA CCA TTG CTT TTG AAG TTG TTA AAG TCC GTT GGA      327
Leu Val Arg Pro Lys Pro Leu Leu Leu Lys Leu Leu Lys Ser Val Gly
                30                  35                  40

GCG CAA AAC GAC ACT TAC ACT ATG AAA GAG ATT ATA TTT TAT ATT GGC      375
Ala Gln Asn Asp Thr Tyr Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly
            45                  50                  55

CAG TAT ATT ATG ACT AAG AGG TTA TAT GAC GAG AAG CAG CAG CAC ATT      423
Gln Tyr Ile Met Thr Lys Arg Leu Tyr Asp Glu Lys Gln Gln His Ile
        60                  65                  70

GTG TAT TGT TCA AAT GAT CTC CTA GGA GAT GTG TTT GGA GTC CCG AGT      471
Val Tyr Cys Ser Asn Asp Leu Leu Gly Asp Val Phe Gly Val Pro Ser
    75                  80                  85                  90

TTC TCT GTG AAG GAG CAC AGG AAA ATA TAT GCA ATG ATC TAC AGA AAT      519
Phe Ser Val Lys Glu His Arg Lys Ile Tyr Ala Met Ile Tyr Arg Asn
                95                  100                 105

TTA GTG GCT GTA AGT CAG CAA GAC TCT GGC ACA TCG CTG AGT GAG AGC      567
Leu Val Ala Val Ser Gln Gln Asp Ser Gly Thr Ser Leu Ser Glu Ser
            110                 115                 120

AGA CGT CAG CCT GAA GGT GGG AGT GAT CTG AAG GAT CCT TTG CAA GCG      615
Arg Arg Gln Pro Glu Gly Gly Ser Asp Leu Lys Asp Pro Leu Gln Ala
        125                 130                 135
```

CCA CCA GAA GAG AAA CCT TCA TCT TCT GAT TTA ATT TCT AGA CTG TCT 663
Pro Pro Glu Glu Lys Pro Ser Ser Ser Asp Leu Ile Ser Arg Leu Ser
140 145 150

ACC TCA TCT AGA AGG AGA TCC ATT AGT GAG ACA GAA GAG AAC ACA GAT 711
Thr Ser Ser Arg Arg Arg Ser Ile Ser Glu Thr Glu Glu Asn Thr Asp
155 160 165 170

GAG CTA CCT GGG GAG CGG CAC CGG AAG CGC CGC AGG TCC CTG TCC TTT 759
Glu Leu Pro Gly Glu Arg His Arg Lys Arg Arg Arg Ser Leu Ser Phe
175 180 185

GAT CCG AGC CTG GGT CTG TGT GAG CTG AGG GAG ATG TGC AGC GGC GGC 807
Asp Pro Ser Leu Gly Leu Cys Glu Leu Arg Glu Met Cys Ser Gly Gly
190 195 200

ACG AGC AGC AGT AGC AGC AGC AGC AGC GAG TCC ACA GAG ACG CCC TCG 855
Thr Ser Ser Ser Ser Ser Ser Ser Ser Glu Ser Thr Glu Thr Pro Ser
205 210 215

CAT CAG GAT CTT GAC GAT GGC GTA AGT GAG CAT TCT GGT GAT TGC CTG 903
His Gln Asp Leu Asp Asp Gly Val Ser Glu His Ser Gly Asp Cys Leu
220 225 230

GAT CAG GAT TCA GTT TCT GAT CAG TTT AGC GTG GAA TTT GAA GTT GAG 951
Asp Gln Asp Ser Val Ser Asp Gln Phe Ser Val Glu Phe Glu Val Glu
235 240 245 250

TCT CTG GAC TCG GAA GAT TAC AGC CTG AGT GAC GAA GGG CAC GAG CTC 999
Ser Leu Asp Ser Glu Asp Tyr Ser Leu Ser Asp Glu Gly His Glu Leu
255 260 265

TCA GAT GAG GAT GAT GAG GTC TAT CGG GTC ACA GTC TAT CAG ACA GGA 1047
Ser Asp Glu Asp Asp Glu Val Tyr Arg Val Thr Val Tyr Gln Thr Gly
270 275 280

GAA AGC GAT ACA GAC TCT TTT GAA GGA GAT CCT GAG ATT TCC TTA GCT 1095
Glu Ser Asp Thr Asp Ser Phe Glu Gly Asp Pro Glu Ile Ser Leu Ala
285 290 295

GAC TAT TGG AAG TGT ACC TCA TGC AAT GAA ATG AAT CCT CCC CTT CCA 1143
Asp Tyr Trp Lys Cys Thr Ser Cys Asn Glu Met Asn Pro Pro Leu Pro
300 305 310

TCA CAC TGC AAA AGA TGC TGG ACC CTT CGT GAG AAC TGG CTT CCA GAC 1191
Ser His Cys Lys Arg Cys Trp Thr Leu Arg Glu Asn Trp Leu Pro Asp
315 320 325 330

GAT AAG GGG AAA GAT AAA GTG GAA ATC TCT GAA AAA GCC AAA CTG GAA 1239
Asp Lys Gly Lys Asp Lys Val Glu Ile Ser Glu Lys Ala Lys Leu Glu
335 340 345

AAC TCA GCT CAG GCA GAA GAA GGC TTG GAT GTG CCT GAT GGC AAA AAG 1287
Asn Ser Ala Gln Ala Glu Glu Gly Leu Asp Val Pro Asp Gly Lys Lys
350 355 360

CTG ACA GAG AAT GAT GCT AAA GAG CCA TGT GCT GAG GAG GAC AGC GAG 1335
Leu Thr Glu Asn Asp Ala Lys Glu Pro Cys Ala Glu Glu Asp Ser Glu
365 370 375

GAG AAG GCC GAA CAG ACG CCC CTG TCC CAG GAG AGT GAC GAC TAT TCC 1383
Glu Lys Ala Glu Gln Thr Pro Leu Ser Gln Glu Ser Asp Asp Tyr Ser
380 385 390

CAA CCA TCG ACT TCC AGC AGC ATT GTT TAT AGC AGC CAA GAA AGC GTG 1431
Gln Pro Ser Thr Ser Ser Ser Ile Val Tyr Ser Ser Gln Glu Ser Val
395 400 405 410

AAA GAG TTG AAG GAG GAA ACG CAG CAC AAA GAC GAG AGT GTG GAA TCT 1479
Lys Glu Leu Lys Glu Glu Thr Gln His Lys Asp Glu Ser Val Glu Ser
415 420 425

AGC TTC TCC CTG AAT GCC ATC GAA CCA TGT GTG ATC TGC CAG GGG CGG 1527
Ser Phe Ser Leu Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg
430 435 440

CCT AAA AAT GGC TGC ATT GTT CAC GGC AAG ACT GGA CAC CTC ATG TCA 1575
Pro Lys Asn Gly Cys Ile Val His Gly Lys Thr Gly His Leu Met Ser
445 450 455

TGT TTC ACG TGT GCA AAG AAG CTA AAA AAA AGA AAC AAG CCC TGC CCA 1623
Cys Phe Thr Cys Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro
460 465 470

GTG TGC AGA CAG CCA ATC CAA ATG ATT GTG CTA AGT TAC TTC AAC 1668
Val Cys Arg Gln Pro Ile Gln Met Ile Val Leu Ser Tyr Phe Asn
475 480 485

TAGCTGACCT GCTCACAAAA ATAGAATTTT ATATTTCTAA CT 1710

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 489 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Cys Asn Thr Asn Met Ser Val Ser Thr Glu Gly Ala Ala Ser Thr
1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Asn Asp Thr Tyr
35 40 45

Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly Gln Tyr Ile Met Thr Lys
50 55 60

Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys Ser Asn Asp
65 70 75 80

Leu Leu Gly Asp Val Phe Gly Val Pro Ser Phe Ser Val Lys Glu His
85 90 95

Arg Lys Ile Tyr Ala Met Ile Tyr Arg Asn Leu Val Ala Val Ser Gln
100 105 110

Gln Asp Ser Gly Thr Ser Leu Ser Glu Ser Arg Arg Gln Pro Glu Gly
115 120 125

Gly Ser Asp Leu Lys Asp Pro Leu Gln Ala Pro Pro Glu Glu Lys Pro
130 135 140

Ser Ser Ser Asp Leu Ile Ser Arg Leu Ser Thr Ser Ser Arg Arg Arg
145 150 155 160

Ser Ile Ser Glu Thr Glu Glu Asn Thr Asp Glu Leu Pro Gly Glu Arg
165 170 175

His Arg Lys Arg Arg Arg Ser Leu Ser Phe Asp Pro Ser Leu Gly Leu
180 185 190

Cys Glu Leu Arg Glu Met Cys Ser Gly Gly Thr Ser Ser Ser Ser Ser
195 200 205

Ser Ser Ser Glu Ser Thr Glu Thr Pro Ser His Gln Asp Leu Asp Asp
210 215 220

Gly Val Ser Glu His Ser Gly Asp Cys Leu Asp Gln Asp Ser Val Ser
225 230 235 240

Asp Gln Phe Ser Val Glu Phe Glu Val Glu Ser Leu Asp Ser Glu Asp
245 250 255

Tyr Ser Leu Ser Asp Glu Gly His Glu Leu Ser Asp Glu Asp Asp Glu
260 265 270

Val Tyr Arg Val Thr Val Tyr Gln Thr Gly Glu Ser Asp Thr Asp Ser
275 280 285

Phe Glu Gly Asp Pro Glu Ile Ser Leu Ala Asp Tyr Trp Lys Cys Thr
290 295 300

Ser Cys Asn Glu Met Asn Pro Pro Leu Pro Ser His Cys Lys Arg Cys
305 310 315 320

Trp Thr Leu Arg Glu Asn Trp Leu Pro Asp Asp Lys Gly Lys Asp Lys
325 330 335

Val Glu Ile Ser Glu Lys Ala Lys Leu Glu Asn Ser Ala Gln Ala Glu
340 345 350

Glu Gly Leu Asp Val Pro Asp Gly Lys Lys Leu Thr Glu Asn Asp Ala
355 360 365

Lys Glu Pro Cys Ala Glu Glu Asp Ser Glu Glu Lys Ala Glu Gln Thr
370 375 380

Pro Leu Ser Gln Glu Ser Asp Asp Tyr Ser Gln Pro Ser Thr Ser Ser
385 390 395 400

Ser Ile Val Tyr Ser Ser Gln Glu Ser Val Lys Glu Leu Lys Glu Glu
405 410 415

Thr Gln His Lys Asp Glu Ser Val Glu Ser Ser Phe Ser Leu Asn Ala
420 425 430

Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly Cys Ile
435 440 445

Val His Gly Lys Thr Gly His Leu Met Ser Cys Phe Thr Cys Ala Lys
450 455 460

Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln Pro Ile
465 470 475 480

Gln Met Ile Val Leu Ser Tyr Phe Asn
485

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 10, line 19 of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet ☐ [1]

Name of depositary institution [1]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [1]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [1]	Accession Number [1]
March 11, 1993	HB 11290

B. ADDITIONAL INDICATIONS [1] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

Hybridoma: IF2

In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [1] (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [1] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [1] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

M. Holmes
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [1]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 10, line 19 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet [X]

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852, USA

Date of deposit	Accession Number
March 11, 1993	HB 11290

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

IF2 - Hybridoma

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

M. Holmes

(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 10, line 19 of the description [1]

A. IDENTIFICATION OF DEPOSIT [3]

Further deposits are identified on an additional sheet [ ] [4]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [5]

12301 Parklawn Drive
Rockville, Maryland 20852, USA

Date of deposit [6]	Accession Number [7]
March 11, 1993	HB 11291

B. ADDITIONAL INDICATIONS [1] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

ED9 - Hybridoma

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [2] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

M. Holmes

(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT RO/134 (January 1981)

CLAIMS

1. A method of diagnosing a neoplastic tissue in a human comprising:

detecting amplification of human MDM2 gene or elevated expression of a human MDM2 gene product in a tissue or body fluid isolated from a human, wherein amplification of the human MDM2 gene or elevated expression of human MDM2 gene product provides a diagnosis of neoplasia or the potential for neoplastic development.

2. The method of claim 1 wherein gene amplification is detected.

3. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being mRNA.

4. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being human MDM2 protein.

5. The method of claim 3 wherein said mRNA is detected by Northern blot analysis by hybridizing mRNA from said tissue to a human MDM2 nucleotide probe.

6. The method of claim 5 wherein the human MDM2 nucleotide probe comprises nucleotides 1-2372 of human MDM2, as shown in Figure 1, or fragments thereof consisting of at least 14 contiguous nucleotides.

7. The method of claim 4 wherein human MDM2 protein is detected by Western Blot analysis by reacting human MDM2 proteins with antibodies which are immunospecific for MDM2 protein.

8. The method of claim 2 wherein the gene amplification is detected using polymerase chain reaction.

9. The method of claim 2 wherein amplification of the human MDM2 gene is detected by Southern blot analysis wherein the human MDM2 gene is hybridized with a nucleotide probe which is complementary to hMDM2 DNA.

10. The method of claim 2 wherein gene amplification is determined by comparing the copy number of hMDM2 in the tissue to the copy number of hMDM2 in a normal tissue of the human.

11. The method of claim 3 wherein elevated expression of mRNA is determined by comparing the amount of hMDM2 mRNA in the tissue to the amount of hMDM2 mRNA in a normal tissue of the human.

12. The method of claim 4 wherein elevated expression of hMDM2 protein is determined by comparing the amount of hMDM2 protein in the tissue to the amount of hMDM2 protein in a normal tissue of the human.

13. The method of claim 2 wherein gene amplification is detected when at least 3-fold more hMDM-2 DNA is observed in the tissue relative to a control sample comprising a normal tissue.

14. The method of claim 3 wherein elevated expression is detected when at least 3-fold more hMDM-2 mRNA is observed in the tissue relative to a control sample comprising a normal tissue.

15. The method of claim 4 wherein elevated expression is detected when at least 3-fold more hMDM2 protein is observed in the tissue relative to a control sample comprising a normal tissue.

16. The method of claim 1 wherein the neoplasia is a sarcoma.

17. The method of claim 16 wherein the sarcoma is a liposarcoma, malignant fibrous histiocytoma, or osteosarcoma.

18. A cDNA molecule comprising nucleotides 1 to 2372, as shown in Figure 1, or fragments thereof, consisting of at least 14 contiguous nucleotides.

19. The cDNA molecule of claim 18 comprising the coding sequence of human MDM2.

20. Human MDM2 protein substantially free of other human proteins.

21. A preparation of antibodies specifically immunoreactive with human MDM2 protein.

22. The preparation of claim 21 wherein the antibodies are monoclonal antibodies.

23. A nucleotide probe comprising a sequence of at least 10 nucleotides which are complementary to nucleotides 1-2372 of human MDM2 gene, as shown in Figure 1.

24. A kit for detecting the amplification of a human MDM2 gene in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said human MDM2 gene under conditions of high stringency, and instructions for determining said amplification.

25. A kit for detecting elevated expression of a human MDM2 mRNA in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said mRNA, and written instructions for determining elevated expression of mRNA.

26. A kit for detecting elevated expression of a human MDM2 protein in a human tissue or body fluid sample comprising MDM2 protein-specific antibodies and written instructions for determining elevated expression of human MDM2 protein.

27. A method of treating a neoplastic cell or a cell having neoplastic potential, comprising:

administering to a cell a therapeutically effective amount of an inhibitory compound which interferes with the expression of human MDM2 gene.

28. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering antisense oligonucleotides.

29. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering triple-strand forming oligonucleotides which interact with DNA.

30. A method for identifying compounds which interfere with the binding of human MDM-2 to human p53, comprising:

binding a predetermined quantity of a first human protein which is detectably labelled to a second human protein;

adding a compound to be tested for its capacity to inhibit binding of said first and second proteins to each other;

determining the quantity of the first human protein which is displaced from or prevented from binding to the second human protein;

wherein the first human protein is MDM-2 and the second human protein is p53 or the first human protein is p53 and the second human protein is MDM-2.

31. The method of claim 30 wherein one of said two human proteins is fixed to a solid support.

32. The method of claim 30 wherein an antibody specifically immunoreactive with said second human protein is used to separate first human protein bound from unbound first human protein.

33. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering a polypeptide to tumor cells which contain a human MDM2 gene amplification, said polyptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

34. The method of claim 33 wherein said polypeptide comprises amino acids 1-41 of p53.

35. The method of claim 33 wherein said polypeptide comprises amino acids 13-57 of p53.

36. The method of claim 33 wherein said polypeptide comprises amino acids 1-50 of p53.

37. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

38. The method of claim 37 wherein said polypeptide comprises amino acids 1-41 of p53.

39. The method of claim 37 wherein said polypeptide comprises amino acids 13-57 of p53.

40. The method of claim 37 wherein said polypeptide comprises amino acids 1-50 of p53.

41. A polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide capable of binding to human MDM2.

42. The polypeptide of claim 41 which comprises amino acids 1-41 of p53.

43. The polypeptide of claim 41 which comprises amino acids 13-57 of p53.

44. The polypeptide of claim 41 which comprises amino acids 1-50 of p53.

45. The preparation of claim 21 wherein the antibodies do not bind to other human proteins.

46. The preparation of claim 21 wherein the antibodies do not bind to human proteins of M_r 75-85K, 105-120K, and 170-200K.

47. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).

48. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma ED9 (ATCC HB 11291).

49. The method of claim 7 wherein the antibodies bind to the epitope on hMDM2 bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).

50. The method of claim 4 wherein human MDM2 protein is detected by immunohistochemistry.

51. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by ED9 (ATCC HB 11291).

52. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by IF2 (ATCC HB 11290).

53. The method of claim 4 wherein human MDM2 protein is detected by immunoprecipitation.

54. A hybridoma cell having the identifying characteristics of ED9 (ATCC HB 11291).

55. A hybridoma cell having the identifying characteristics of IF2 (ATCC HB 11290).

FIG. 1A(1)

```
   1   GCACCGCGCGAGCTTGGCTGCTTCTGGGGC

                                     * AG
  84   GGCCGCGACCCCTCTGACCGAGATCCTGCTG

       CGT  GC  GG CTCCGCGCTCCCCG GAAG
 168   GTGCCCTGGCCCGGAGAGTGGAATGATCCCC

       ACC GACACCCCTGGGGGACC   TCG AT
 252   GGAGTCTTGAGGGACCCCCGACTCCAAGCGC
   1

       T    C  G        C  G
 336   CCTACTGATGGTGCTGTAACCACCTCACAGA
   9    P  T  D  G  A  V  T  T  S  Q
        S     E        A  S

         G         C     A  G     C
 420   TTATTAAAGTCTGTTGGTGCACAAAAAGACA
  37    L  L  K  S  V  G  A  Q  K  D
                                  N

       A G         C         G  G  C
 504   CGATTATATGATGAGAAGCAACAACATATTG
  65    R  L  Y  D  E  K  Q  Q  H  I

           G                     G A
 588   GTGAAAGAGCACAGGAAAATATATACCATGA
  93    V  K  E  H  R  K  I  Y  T  M
                                  A

         GC          G   AC    G C
 672   TCTGTGAGTGAGAACAGGTGTCACCTTGAAG
 121    S  V  S  E  N  R  C  H  L  E
           L        S     R  Q  P
```

FIG. 1A(2)

```
CTGTGTGGCCCTGTGTGTCGGAAAGATGGAGCAAGA

AGCCGC GC TTCTC TCG TCGAGCT TG ACGAC
CTTTCGCAGCCAGGAGCACCGTCCCTCCCCGGATTA

 GTCGGAA ATGCGC G AAGTAG   CC   T CT
GAGGCCCAGGGCGTCGTGCTTCCGCAGTAGTCAGTC

ACCGCG TTCTCCT C GCCTC     C
GAAAACCCCGGATGGTGAGGAGCAGGCAAATGTGCA
                                M  C


                          T
TTCCAGCTTCGGAACAAGAGACCCTGGTTAGACCAA
I  P  A  S  E  Q  E  T  L  V  R  P


    C              A  A A      A
CTTATACTATGAAAGAGGTTCTTTTTTATCTTGGCC
T  Y  T  M  K  E  V  L  F  Y  L  G
                  I  I        I

 G                    C         G
TATATTGTTCAAATGATCTTCTAGGAGATTTGTTTG
V  Y  C  S  N  D  L  L  G  D  L  F
                                  V

       A  T  A  G CT  A G       A----
TCTACAGGAACTTGGTAGTAGTCAATCAGCAGGAAT
I  Y  R  N  L  V  V  V  N  Q  Q  E
                  A     S        -

            TG     T C T G     C  CA
GTGGGAGTGATCAAAAGGACCTTGTACAAGAGCTTC
G  G  S  D  Q  K  D  L  V  Q  E  L
            L        P  L     A  P
```

FIG. 1A(3)

```
AGCCGAGCCCGAGGGGC          83   Human nt

CATG  CGCTCA G  C               Mouse nt
GTGCGTACGAGCGCCCA         167   Human nt

GGGCGAGC  GAGACC                Mouse nt
CCCGTGAAGGAAACTGG         251   Human nt

                  G             Mouse nt
ATACCAACATGTCTGTA         335   Human nt
N  T  N  M  S  V            8   Human a.a.
                                Mouse a.a.

 A                              Mouse nt
AGCCATTGCTTTTGAAG         419   Human nt
K  P  L  L  L  K           36   Human a.a.
                                Mouse a.a.

                  G             Mouse nt
AGTATATTATGACTAAA         503   Human nt
Q  Y  I  M  T  K           64   Human a.a.
                                Mouse a.a.

 A  C  G  T                     Mouse nt
GCGTGCCAAGCTTCTCT         587   Human nt
G  V  P  S  F  S           92   Human a.a.
                                Mouse a.a.

-----     T  C                 Mouse nt
CATCGGACTCAGGTACA         671   Human nt
S  S  D  S  G  T          120   Human a.a.
-  -                            Mouse a.a.

CA                              Mouse nt
AGGAAGAGAAACCTTCA         755   Human nt
Q  E  E  K  P  S          148   Human a.a.
P                               Mouse a.a.
```

FIG. 1B(1)

```
          TG     AA          TG
756  TCTTCACATTTGGTTTCTAGACCATCT
149   S  S  H  L  V  S  R  P  S
         D     I        L

       G  G  G  CC G  G     G  GG
840  GGTGAACGACAAAGAAAACGCCACAAA
177   G  E  R  Q  R  K  R  H  K
            H              R  R

       G  CAGCGGCGGCACGAGCA CAGT
924  ATATGT----------------TGTGAA
205   I  C  -  -  -  -  -  C  E
      M     S  G  G  T  S  S  S

            G     T         CC
993  GTAAGTGAACATTCAGGTGATTGGTTG
228   V  S  E  H  S  G  D  W  L
                           C

       G        C     G     C
1077 TCAGAAGATTATAGCCTTAGTGAAGAA
256   S  E  D  Y  S  L  S  E  E
                           D

       A  A  C        C  T
1161 GGGGAGAGTGATACAGATTCATTTGAA
284   G  E  S  D  T  D  S  F  E

          T                 C     A
1245 AATCCCCCCCTTCCATCACATTGCAAC
312   N  P  P  L  P  S  H  C  N
                              K

             A
1329 GAAATCTCTGAGAAAGCCAAACTGGAA
340   E  I  S  E  K  A  K  L  E
```

FIG. 1B(2)

```
                        T C                         G
ACCTCATCTAGAAGGAGAGCAATTAGTGAGACAGAAGAA
 T  S  S  R  R  R  A  I  S  E  T  E  E
                     S

------------      G          CCG        G
TCTGATAGTATTTCCCTTTCCTTTGATGAAAGCCTGGCT
 S  D  S  I  S  L  S  F  D  E  S  L  A
 -  -  -  -                 P        G

  C     C     C  G  C     A     C  C
AGAAGCAGTAGCAGTGAATCTACAGGGACGCCATCGAAT
 R  S  S  S  S  E  S  T  G  T  P  S  N
 S                       E           H

                     T           C  G
GATCAGGATTCAGTTTCAGATCAGTTTAGTGTAGAATTT
 D  Q  D  S  V  S  D  Q  F  S  V  E  F

  G  C  G           G           C    GG
GGACAAGAACTCTCAGATGAAGATGATGAGGTATATCAA
 G  Q  E  L  S  D  E  D  D  E  V  Y  Q
    H                                R

 G        G                          G  T
GAAGATCCTGAAATTTCCTTAGCTGACTATTGGAAATGC
 E  D  P  E  I  S  L  A  D  Y  W  K  C
 G

    C  A              C        A  C
AGATGTTGGGCCCTTCGTGAGAATTGGCTTCCTGAAGAT
 R  C  W  A  L  R  E  N  W  L  P  E  D
          T                       D

      G T  G  A     A     G     G
AACTCAACACAAGCTGAAGAGGGCTTTGATGTTCCTGAT
 N  S  T  Q  A  E  E  G  F  D  V  P  D
       A                 L
```

FIG. 1B(3)

```
  CA         GC  C                          Mouse nt
AATTCAGATGAATTATCT      839                 Human nt
 N  S  D  E  L  S       176                 Human a.a.
    T           P                           Mouse a.a.

       AGC G                                Mouse nt
CTGTGTGTAATAAGGGAG      923                 Human nt
 L  C  V  I  R  E       204                 Human a.a.
       E  L                                 Mouse a.a.

 A          C A  C                          Mouse nt
CCGGATCTTGATGCTGGT      992                 Human nt
 P  D  L  D  A  G       227                 Human a.a.
 Q           D                              Mouse a.a.

        G     G                             Mouse nt
GAAGTTGAATCTCTCGAC     1076                 Human nt
 E  V  E  S  L  D       255                 Human a.a.
                                            Mouse a.a.

  C  A  C       A                           Mouse nt
GTTACTGTGTATCAGGCA     1160                 Human nt
 V  T  V  Y  Q  A       283                 Human a.a.
                T                           Mouse a.a.

  C                                         Mouse nt
ACTTCATGCAATGAAATG     1244                 Human nt
 T  S  C  N  E  M       311                 Human a.a.
                                            Mouse a.a.

  G                 T                       Mouse nt
AAAGGGAAAGATAAAGGG     1228                 Human nt
 K  G  K  D  K  G       339                 Human a.a.
                V                           Mouse a.a.

G C     GCTG C  A                          Mouse nt
TGTAAAAAAACTATAGTG     1412                 Human nt
 C  K  K  T  I  V       367                 Human a.a.
 G        L  T  E                          Mouse a.a.
```

FIG. 1C(1)

```
             G T A     C         C         G
1413  AATGATTCCAGAGAGTCATGTGTTGAGGAA
 368   N  D  S  R  E  S  C  V  E  E
          A  K     P     A

        C  A     G     C  C        G
1494  TCTCAGCCATCAACTTCTAGTAGCATTATT
 395   S  Q  P  S  T  S  S  S  I  I
                                   V

        C                      C  CT    G
1578  GAAGAGAGTGTGGAATCTAGTTTGCCCCTT
 423   E  E  S  V  E  S  S  L  P  L
        D                      F  S

        T  C     G  T     C  C   T A
1662  GTCCATGGCAAAACAGGACATCTTATGGCC
 451   V  H  G  K  T  G  H  L  M  A
                                   S

           G     C                  G
1746  AGACAACCAATTCAAATGATTGTGCTAACT
 479   R  Q  P  I  Q  M  I  V  L  T
                                   S

1830  TAACCCTAGGAATTTAGACAACCTGAAATT
1914  TTAGTATAATTGACCTACTTTGGTAGTGGA
1998  ACTCCTAATTTTAAATAATTTCTACTCTGT
2082  ATGTAACTTATTATTTTTTTTGAGACCGAG
2166  CTCTGCCCTCCCCGGGTTCGCACCATTCTC
2250  TAATTTTTTGTACTTTTAGTAGAGACAGGG
2334  CTCGGCCTCCCAAAGTGCTGGGATTACAGG
```

FIG. 1C(2)

```
G CAGC   G   G   GGCCGA    GA GC C TG   C
AAT---GATGATAAAATTACACAAGCTTCACAATCAC
 N   -   D   D   K   I   T   Q   A   S   Q   S
 D   S   E   E       A   E       T   P   L

                    AGC                 G---  A
TATAGCAGCCAAGAAGATGTGAAAGAGTTTGAAAGGG
 Y   S   S   Q   E   D   V   K   E   F   E   R
                     S                   L   -   K

          C       A           C   C   G   G   G
AATGCCATTGAACCTTGTGTGATTTGTCAAGGTCGAC
 N   A   I   E   P   C   V   I   C   Q   G   R

  T   C   G                           A       A   C
TGCTTTACATGTGCAAAGAAGCTAAAGAAAAGGAATA
 C   F   T   C   A   K   K   L   K   K   R   N

  C    AA     C          CTCA A A   T
TATTTCCCCTAGTTGACCTG---TCTATAAGAGAATT
 Y   F   P
         N

TATTCACATATATCAAAGTGAGAAAATGCCTCAATTC
ATAGTGAATACTTACTATAATTTGACTTGAATATGTA
CTTAAATGAGAAGTACTTGGTTTTTTTTTTCTTAAAT
TCTTGCTCTGTTACCCAGGCTGGAGTGCAGTGGGTGA
CTGCCTCAGCCTCCCAATTAGCTTGGCCTACAGTCAT
TTTCACCGTGTTAGCCAGGATGGTCTCGATCTCCTGA
CATGAGCCACCG
```

FIG. 1C(3)

```
 G  G      C                          Mouse nt
AAGAAAGTGAAGACTAT      1493           Human nt
Q  E  S  E  D  Y        394           Human a.a.
         D                            Mouse a.a.

 G     G  GC                          Mouse nt
AAGAAACCCAAGACAAA      1577           Human nt
E  E  T  Q  D  K        422           Human a.a.
            H                        Mouse a.a.

            C                        Mouse nt
CTAAAAATGGTTGCATT      1661           Human nt
P  K  N  G  C  I        450           Human a.a.
                                      Mouse a.a.

               G  C                   Mouse nt
AGCCCTGCCCAGTATGT      1745           Human nt
K  P  C  P  V  C        478           Human a.a.
                                      Mouse a.a.


T              *                      Mouse nt
ATATATTTCTAACTATA      1829           Human nt
                        491           Human a.a.
                                      Mouse a.a.


ACATAGATTTCTTCTCT      1913           Human nt
GCTCATCCTTTACACCA      1997           Human nt
ATGTATATGACATTTAA      2081           Human nt
TCTTGGCTCACTGCAAG      2165           Human nt
CTGCCACCACACCTGGC      2249           Human nt
CCTCGTGATCCGCCCAC      2333           Human nt
                       2372           Human nt
```

FIG. 2

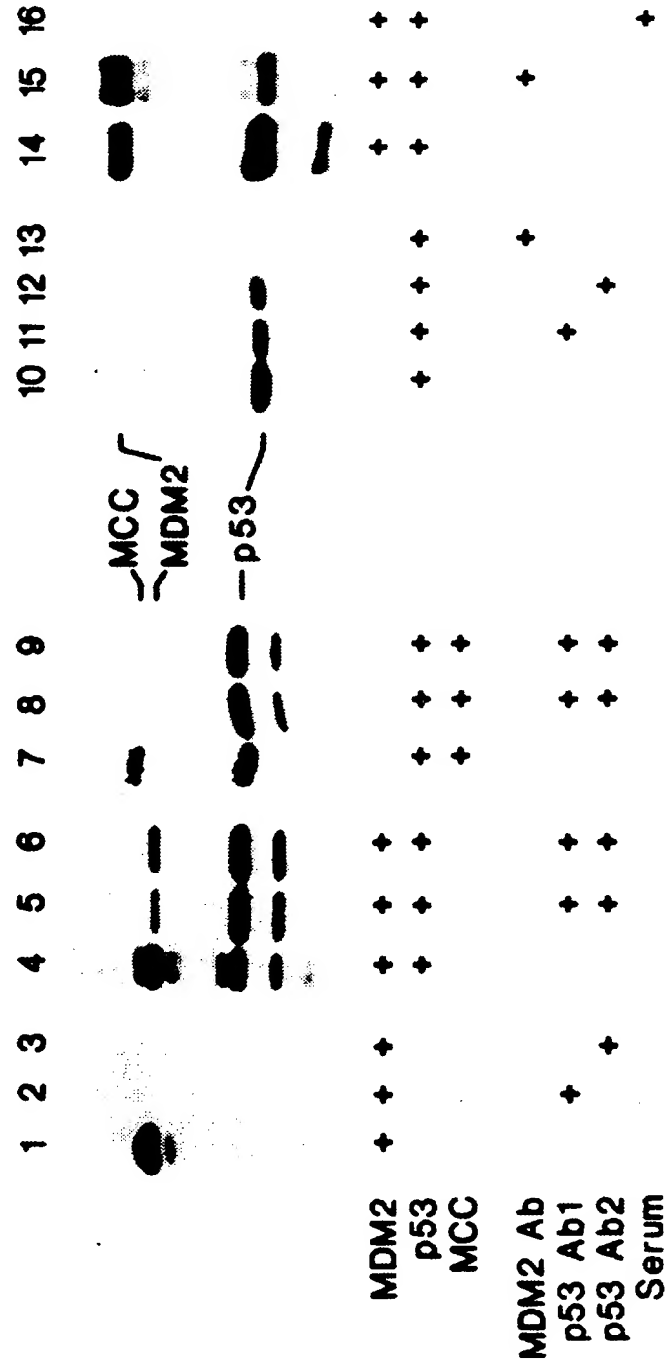

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
MCC
MDM2
p53
MDM2
p53
MCC
MDM2 Ab
p53 Ab1
p53 Ab2
Serum

FIG. 3

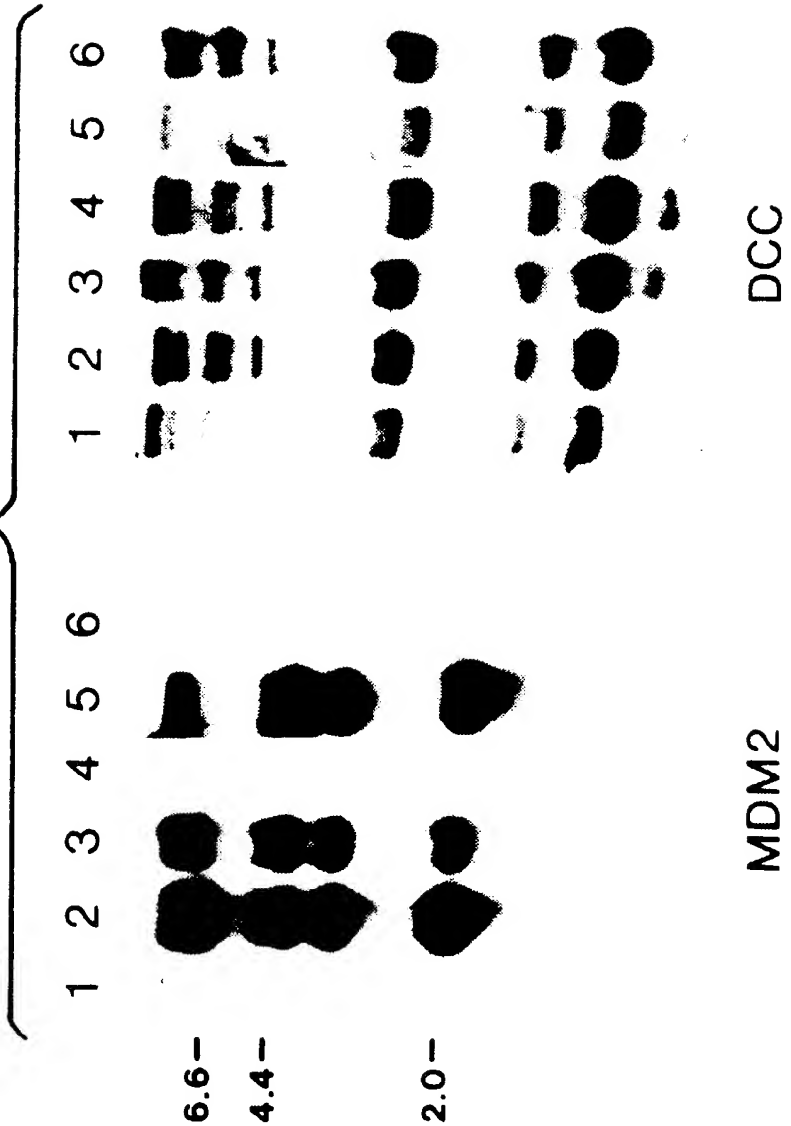


FIG. 4A

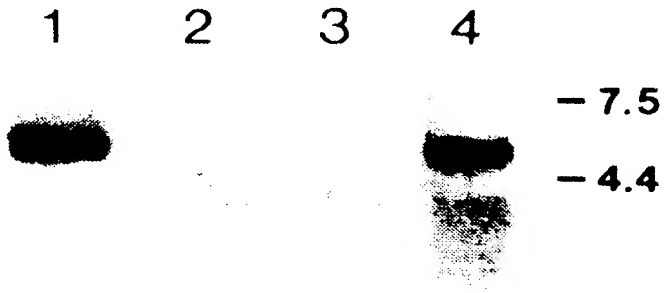


FIG. 4B

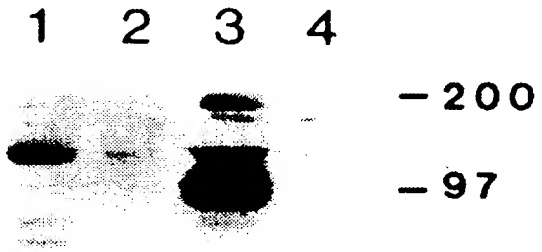


FIG. 4C

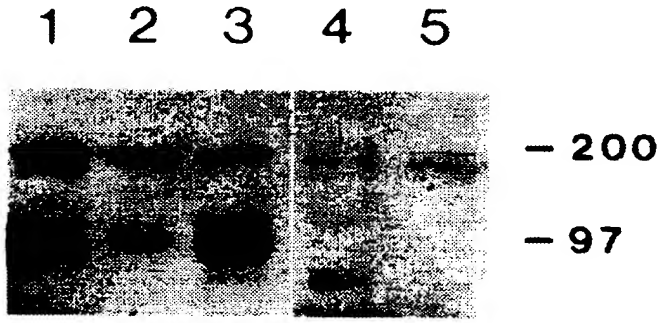
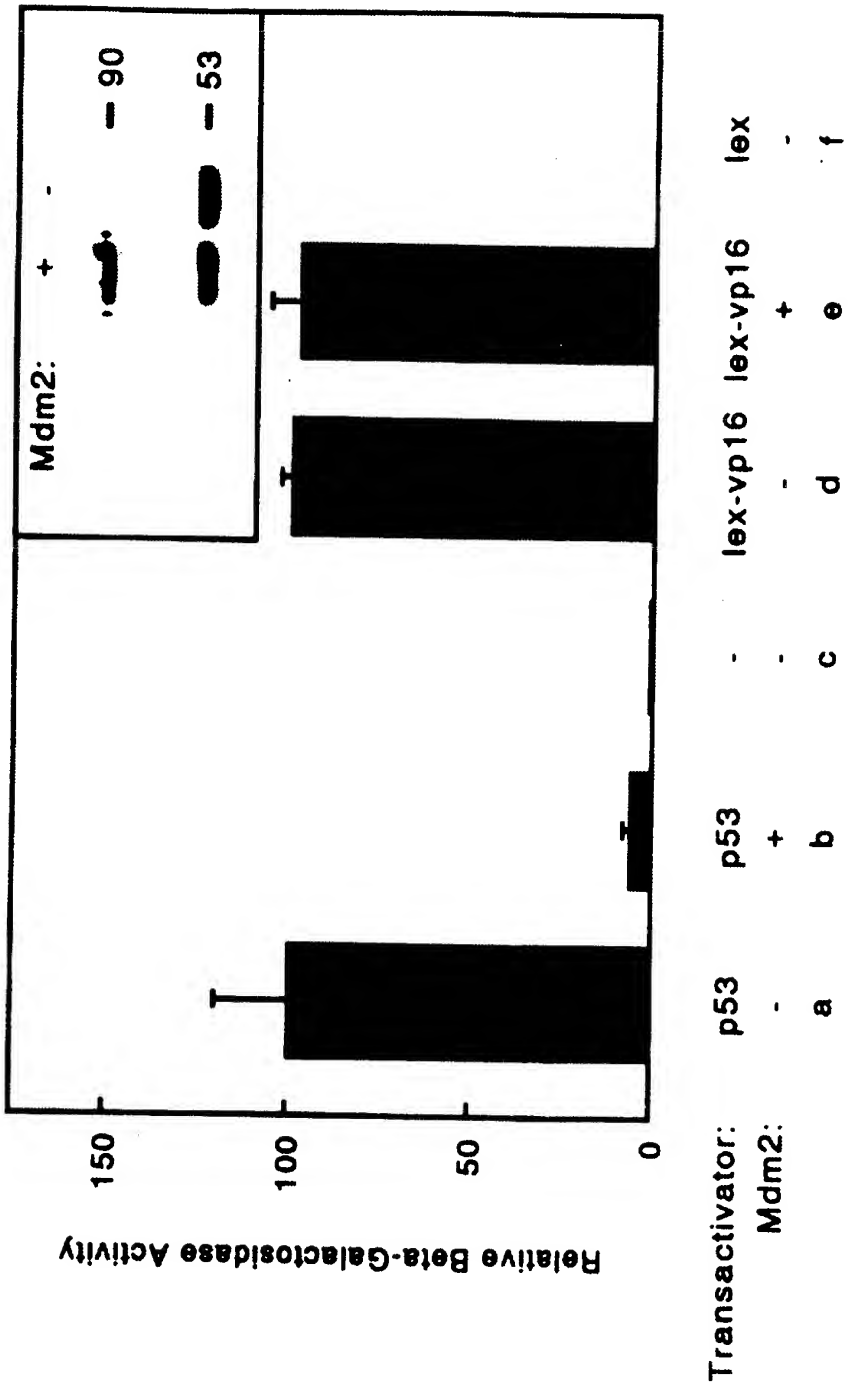


FIG. 5


Mdm2: + -
90
53
150
100
50
0
Relative Beta-Galactosidase Activity
Transactivator: p53 p53 - lex-vp16 lex-vp16 lex
Mdm2: - + - - + -
a b c d e f

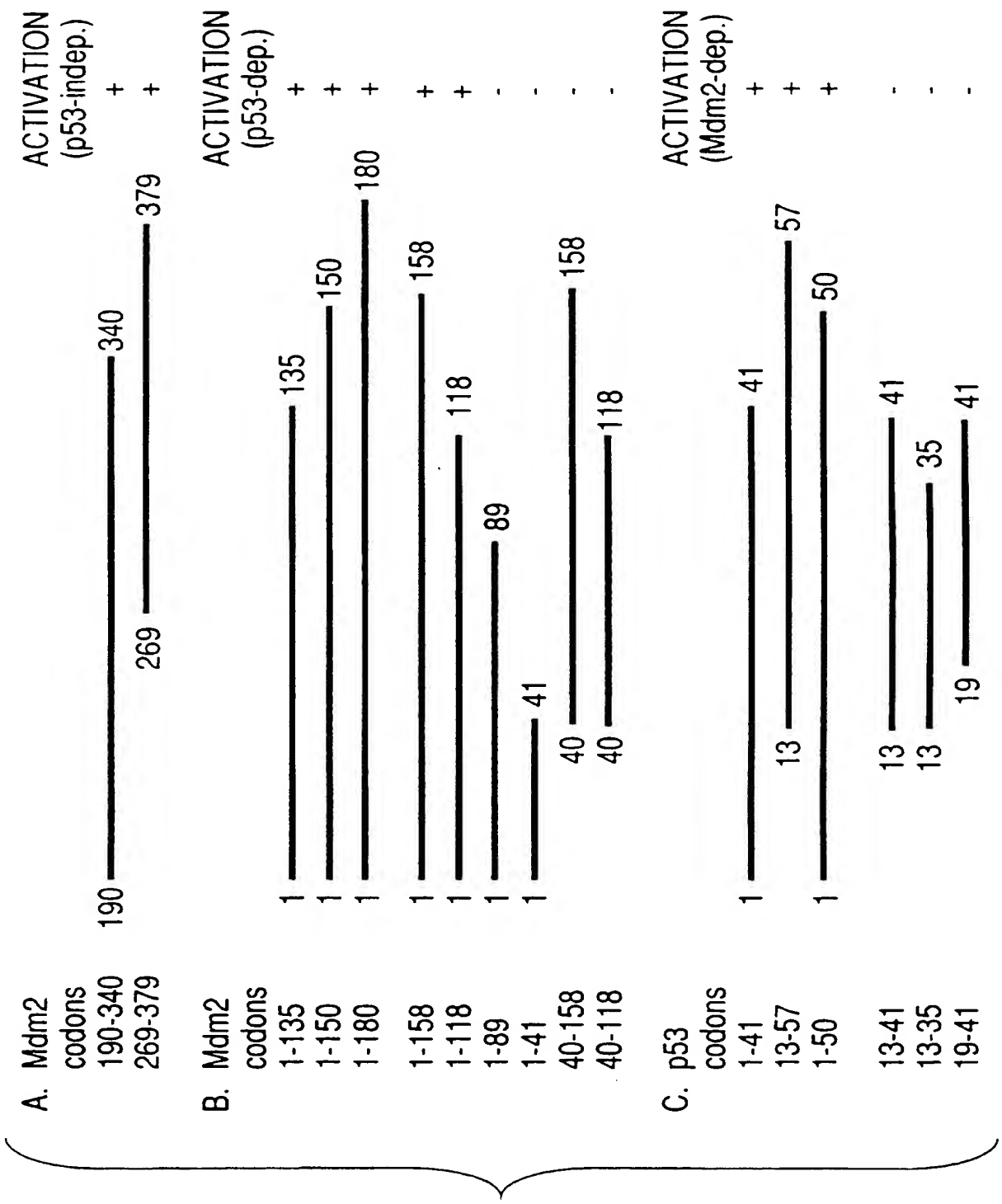

A. Mdm2 codons
190-340
269-379
ACTIVATION (p53-indep.)
+
+
190
340
269
379
B. Mdm2 codons
1-135
1-150
1-180
1-158
1-118
1-89
1-41
40-158
40-118
ACTIVATION (p53-dep.)
+
+
+
+
+
-
-
-
-
1
135
150
180
158
118
89
41
158
118
40
40
C. p53 codons
1-41
13-57
1-50
13-41
13-35
19-41
ACTIVATION (Mdm2-dep.)
+
+
+
-
-
-
1
41
13
57
1
50
13
41
13
35
19
41

FIG. 6

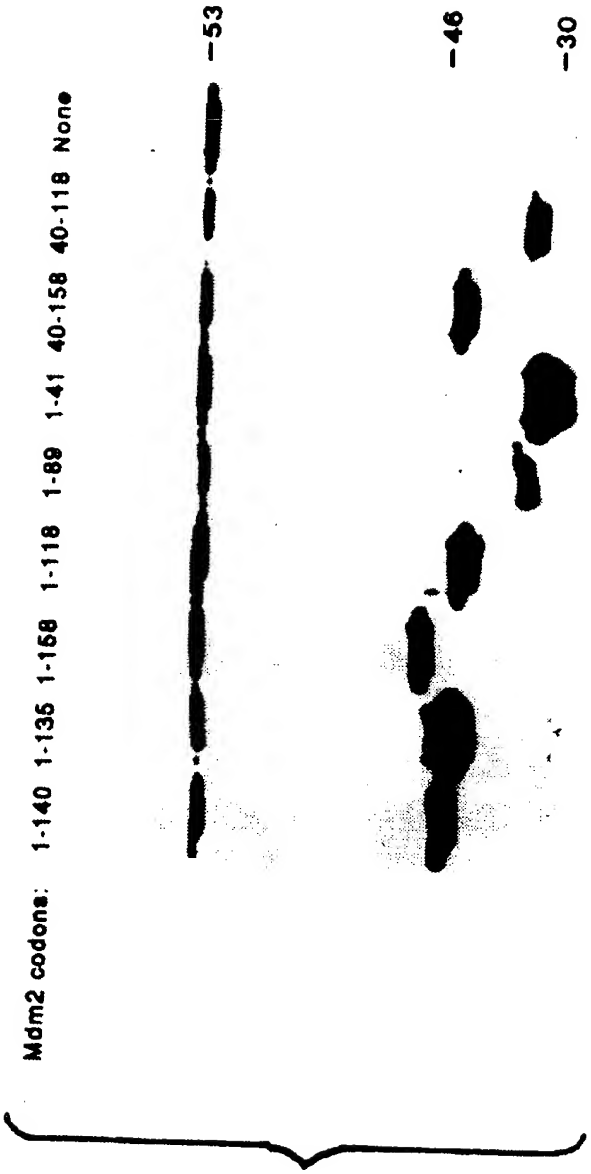

Mdm2 codons: 1-140 1-135 1-168 1-118 1-89 1-41 40-158 40-118 None
-53
-46
-30

FIG. 7A

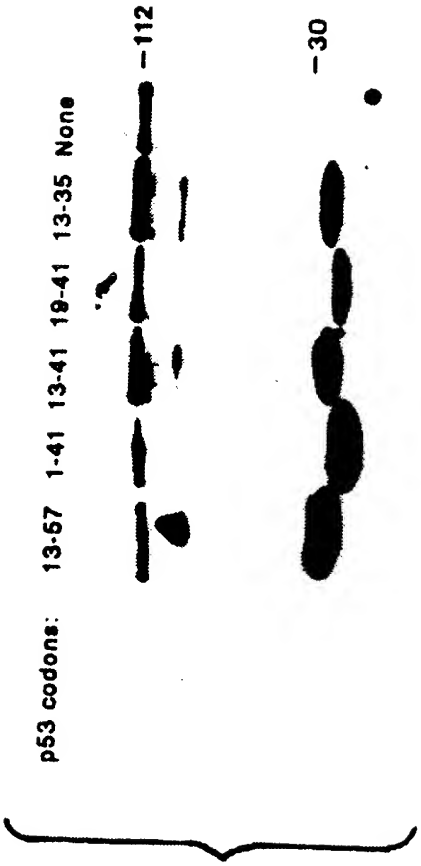

p53 codons: 13-67 1-41 13-41 19-41 13-35 None
-112
-30

FIG. 7B

FIG. 8

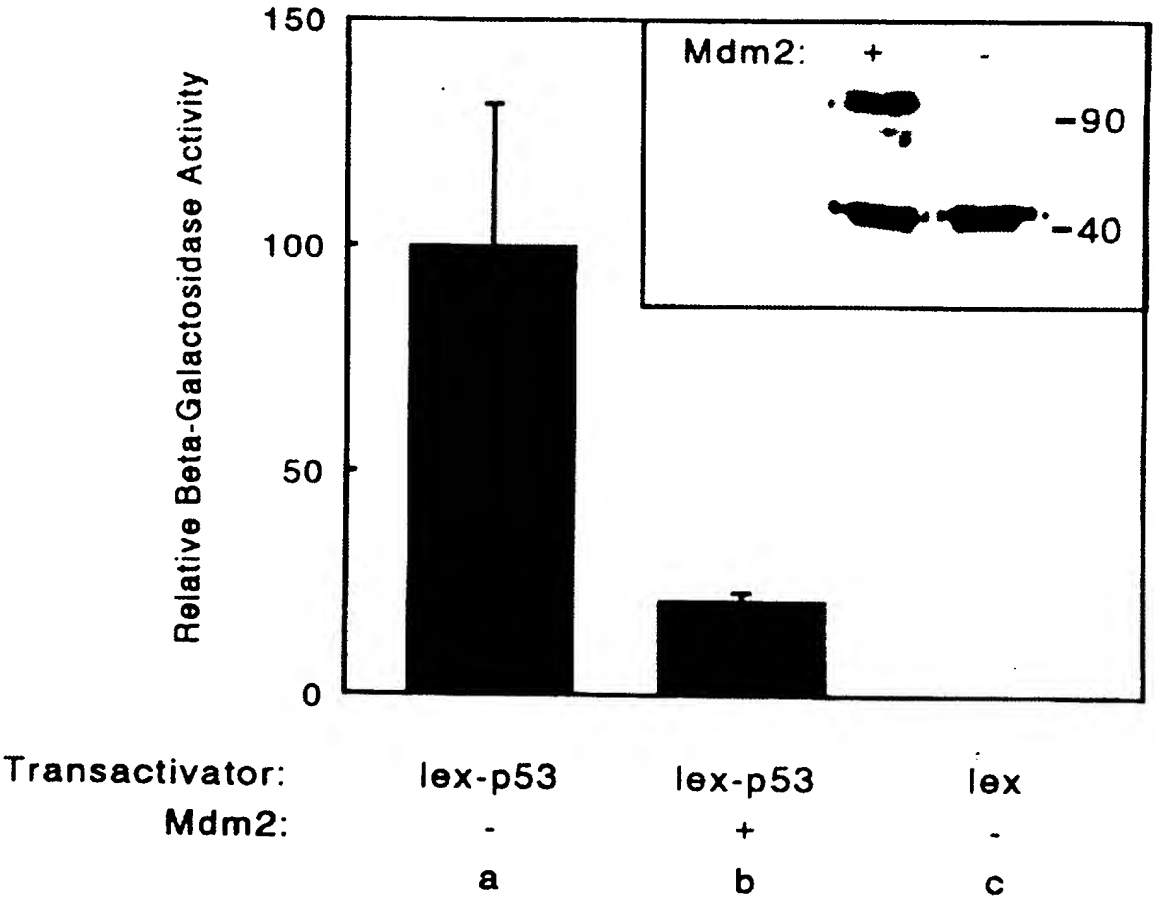

Relative Beta-Galactosidase Activity
150
100
50
0
Mdm2: + -
-90
-40
Transactivator: lex-p53 lex-p53 lex
Mdm2: - + -
a b c

FIGURE 9

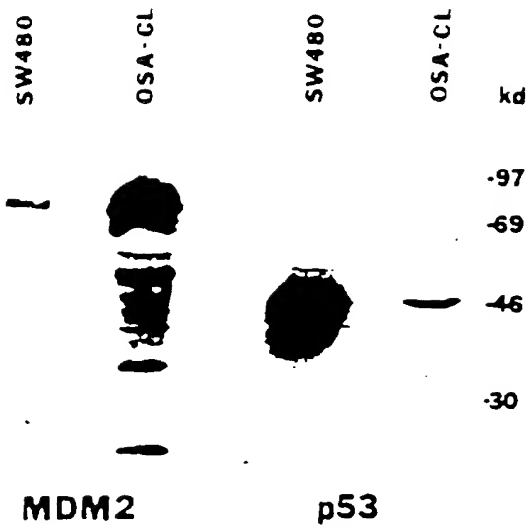


FIGURE 10

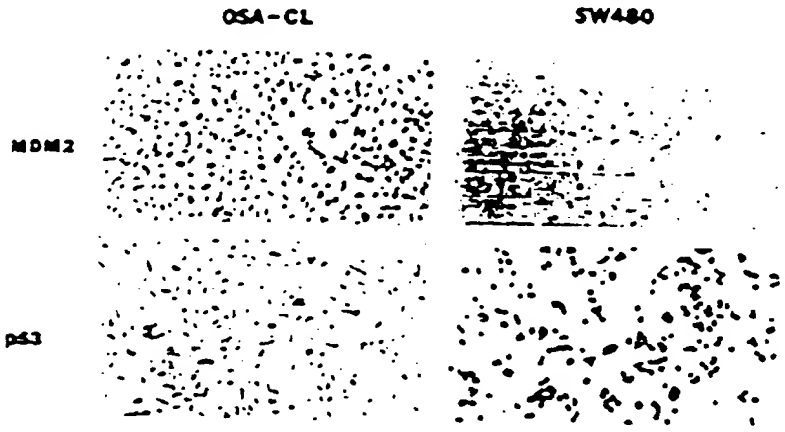
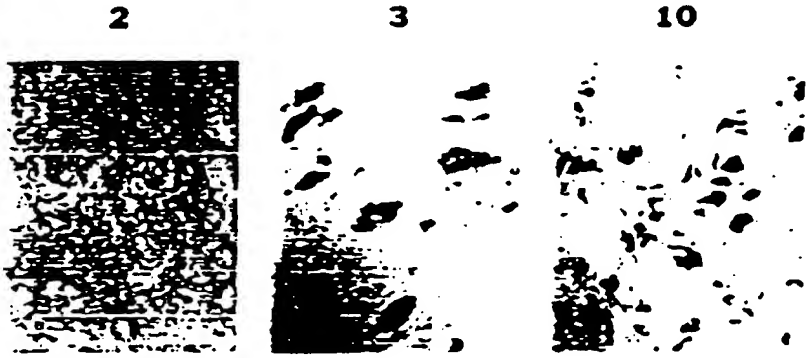


FIGURE 11



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/03199

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; G01N33/574; C07H21/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	EMBO JOURNAL. vol. 10, no. 6, 1991, EYNSHAM, OXFORD GB pages 1565 - 1569 FAKHARZADEH ET AL. 'Tumoriginic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line' cited in the application see the whole document ---	1-55
A	J BIOL CHEM 263 (32). 1988. 17150-17158. SNYDER L C ET AL. 'A GENE AMPLIFIED IN A TRANSFORMED MOUSE CELL LINE UNDERGOES COMPLEX TRANSCRIPTIONAL PROCESSING AND ENCODES A NUCLEAR PROTEIN.' --- -/--	-

* Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 SEPTEMBER 1993	01. 10. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MOLINA GALAN E.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CELL GROWTH & DIFFERENTIATION vol. 1, 1990, pages 571 - 580 HINDS ET AL. 'Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes' cited in the application	-
A	EP,A,0 341 904 (TEMPLE UNIVERSITY) 15 November 1989 see abstract	30-33
A	US,A,4 968 603 (SLAMON ET AL.) 6 November 1990	-
P,X	NATURE vol. 358, 2 July 1992, LONDON GB pages 80 - 83 OLINER ET AL. 'Amplification of a gene encoding a p53 associated protein in human sarcomas' see the whole document	1,18,20
P,X	CELL 69 (7). 1992. 1237-1245. MOMAND J ET AL. 'THE MDM -2 ONCOGENE PRODUCT FORMS A COMPLEX WITH THE'	20

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9303199
SA 73548

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 17/09/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0341904	15-11-89	JP-A- 2013400	17-01-90
US-A-4968603	06-11-90	None	

EPO FORM P079

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

91

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/68, G01N 33/574
C07H 21/00

A3

(11) International Publication Number: WO 93/20238

(43) International Publication Date: 14 October 1993 (14.10.93)

(21) International Application Number: PCT/US93/03199

(22) International Filing Date: 7 April 1993 (07.04.93)

(30) Priority data:

867,840	7 April 1992 (07.04.92)	US
903,103	23 June 1992 (23.06.92)	US

(71) Applicant: THE JOHNS HOPKINS UNIVERSITY [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US).

(72) Inventors: BURRELL, Marilee ; 198 Hampshire Street, Cambridge, MA 02139 (US). HILL, David, E. ; 85 Ridge STreet, Arlington, MA 02174 (US). KINZLER, Kenneth, W. ; 1348 Halstead Road, Baltimore, MA 21234 (US). VOGELSTEIN, Bert ; 3700 Breton Way, Baltimore, MA 21208 (US).

(74) Agents: KAGAN, Sarah, A. et al.; Banner, Birch, McKie & Beckett, 1001 G Street, N.W., 11th Floor, Washington, DC 20001-4597 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
11 November 1993 (11.11.93)

(54) Title: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

(57) Abstract

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to human p53 and allows the cell to escape from p53-regulated growth.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NL**	Netherlands
BE	Belgium	**GN**	Guinea	**NO**	Norway
BF	Burkina Faso	**GR**	Greece	**NZ**	New Zealand
BG	Bulgaria	**HU**	Hungary	**PL**	Poland
BJ	Benin	**IE**	Ireland	**PT**	Portugal
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SK**	Slovak Republic
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SN**	Senegal
CM	Cameroon	**LI**	Liechtenstein	**SU**	Soviet Union
CS	Czechoslovakia	**LK**	Sri Lanka	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**MC**	Monaco	**UA**	Ukraine
DK	Denmark	**MG**	Madagascar	**US**	United States of America
ES	Spain	**ML**	Mali	**VN**	Viet Nam
FI	Finland	**MN**	Mongolia		

AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

This application is a continuation-in-part of United States Serial No. 07/903,103, filed June 23, 1992, which is a continuation-in-part of United States Serial No. 07/867,840, filed April 7, 1992, now abandoned.

This invention was made with support from the U.S. Government, including NIH grants CA-57345, CA-43460, CA-02243 and CA-35494. Accordingly, the Government retains certain rights in the invention.

FIELD OF THE INVENTION

The invention relates to the area of cancer diagnostics and therapeutics. More particularly, the invention relates to the detection of a gene which is amplified in certain human tumors.

BACKGROUND OF THE INVENTION

According to the Knudson model for tumorigenesis (Cancer Research, 1985, vol. 45, p. 1482), there are tumor suppressor genes in all normal cells which, when they become non-functional due to mutation, cause neoplastic development. Evidence for this model has been found in cases of retinoblastoma and colorectal tumors. The implicated suppressor genes in these tumors, RB and p53 respectively, were found to be deleted or altered in many of the tumors studied.

The p53 gene product, therefore, appears to be a member of a group of proteins which regulate normal cellular proliferation and suppression of cellular transformation. Mutations in the p53 gene have been linked to tumorigenesis, suggesting that alterations

in p53 protein function are involved in cellular transformation. The inactivation of the p53 gene has been implicated in the genesis or progression of a wide variety of carcinomas (Nigro et al., 1989, *Nature 342*:705-708), including human colorectal carcinoma (Baker et al., 1989, *Science 244*:217-221), human lung cancer (Takahashi et al., 1989, *Science 246*:491-494; Iggo et al., 1990, *Lancet 335*:675-679), chronic myelogenous leukemia (Kelman et al, 1989, *Proc. Natl. Acad. Sci. USA 86*:6783-6787) and osteogenic sarcomas (Masuda et al., 1987, *Proc. Natl. Acad. Sci. USA 84*:7716-7719).

While there exists an enormous body of evidence linking p53 gene mutations to human tumorigenesis (Hollstein et al., 1991, *Science 253*:49-53) little is known about cellular regulators and mediators of p53 function.

Hinds et al. (*Cell Growth & Differentiation, 1*:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated *ras* oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, *Science 253*:49-53. The transformed fibroblasts were found to produce elevated levels of human p53 protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type human p53 gene does not possess transforming activity in cooperation with *ras*. Tuck and Crawford, 1989, *Oncogene Res. 4*:81-96.

Hinds et al., *supra* also expressed human p53 protein in transformed rat cells. When the expressed human p53 was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified M_r 90,000 protein was coimmunoprecipitated. This suggested that the rat M_r 90,000 protein is in a complex with the human p53 protein in the transformed rat cell line.

As mentioned above, levels of p53 protein are often higher in transformed cells than normal cells. This is due to mutations which increase its metabolic stability (Oven et al., 1981, *Mol. Cell. Biol. 1*:101-110; Reich et al. (1983), *Mol. Cell. Biol. 3*:2143-2150). The stabilization of p53 has been associated with complex formation between p53 and viral or cellular proteins. (Linzer and Levine, 1979, *Cell 17*:43-52; Crawford et al., 1981, *Proc. Natl. Acad. Sci. USA 78*:41-45; Dippold et al., 1981, *Proc. Natl. Acad. Sci. USA 78*:1695-1699; Lane and Crawford, 1979, *Nature (Lond.) 278*:261-263; Hinds et al., 1987, *Mol. Cell. Biol. 7*:2863-2869; Finlay et al., 1988, *Mol. Cell. Biol. 8*:531-539; Sarnow et al., 1982, *Cell. 28*:387-394; Gronostajski et al., 1984, *Mol. Cell. Biol. 4*:442-448; Pinhasi-Kimhi et al., 1986, *Nature (Lond.) 320*:182-185; Ruscetti and Scolnick, 1983, *J. Virol. 46*:1022-1026; Pinhasi and Oren, 1984, *Mol. Cell. Biol. 4*:2180-2186; and Sturzbecher et al., 1987, *Oncogene 1*:201-211.) For example, p53 protein has been observed to form oligomeric protein complexes with the SV40 large T antigen, the adenovirus type 5 E1B-M, 55,000 protein, and the human papilloma virus type 16 or 18 E6 product. Linzer and Levine, 1979, *Cell 17*:43-52; Lane and Crawford, 1979, *Nature, 278*:261-263; Sarnow et al., 1982, *Cell 28*:387-394; Werness et al., 1990, *Science, 248*:76-79. Similarly, complexes have been observed of $p105^{RB}$ (the product of the retinoblastoma susceptibility gene) with T antigen (DeCaprio et al., 1988, *Cell 54*:275-283), the adenovirus E1A protein (Whyte et al., 1988, *Nature 334*:124-129) and the E7 protein of human papilloma virus 16 or 18 (Münger et al., 1989, *EMBO J. 8*:4099-4105). It has been suggested that interactions between these viral proteins and $p105^{RB}$ inactivate a growth-suppressive function of $p105^{RB}$, mimicking deletions and mutations commonly found in the RB gene in tumor cells. In a similar fashion, oligomeric protein complex

formation between these viral proteins and p53 may eliminate or alter the function of p53. Finlay et al., 1989, *Cell 57*:1083-1093.

Fakharzadeh et al. (*EMBO J. 10*:1565-1569, 1991) analyzed amplified DNA sequences present in a tumorigenic mouse cell line (*i.e.*, 3T3DM, a spontaneously transformed derivative of mouse Balb/c cells). Studies were conducted to determine whether any of the amplified genes induced tumorigenicity following introduction of the amplified genes into a nontransformed recipient cell (*e.g.*, mouse NIH3T3 or Rat2 cells). The resulting cell lines were tested for tumorigenicity in nude mice. A gene, designated MDM2, which is amplified more than 50-fold in 3T3DM cells, induced tumorigenicity when overexpressed in NIH3T3 and Rat 2 cells. From the nucleotide and predicted amino acid sequence of mouse MDM2 (mMDM2), Fakharzadeh speculated that this gene encodes a potential DNA binding protein that functions in the modulation of expression of other genes and, when present in excess, interferes with normal constraints on cell growth.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for diagnosing a neoplastic tissue, such as sarcoma, in a human.

It is another object of the invention to provide a cDNA molecule encoding the sequence of human MDM2.

Yet another object of the invention is to provide a preparation of human MDM2 protein which is substantially free of other human cellular proteins.

Still another object of the invention is to provide DNA probes capable of hybridizing with human MDM2 genes or mRNA molecules.

Another object of the invention is to provide antibodies immunoreactive with human MDM2 protein.

Still another object of the invention is to provide kits for detecting amplification or elevated expression of human MDM2.

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method of treating a neoplastic human cell.

Yet another object of the invention is to provide methods for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification.

Still another object of the invention is to provide polypeptides which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method for growing host cells containing a p53 expression vector.

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to human p53 *in vitro*. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C shows the cDNA sequence of human MDM2. In this figure, human and mouse nucleotide and amino acid sequences are compared, the mouse sequence being shown only where it differs from the corresponding human sequence.

Figure 2 shows that hMDM2 binds to p53.

Figure 3 illustrates the amplification of the hMDM2 gene in sarcomas.

Figure 4A-C illustrates hMDM2 expression.

Figure 5 shows the inhibition of p53-mediated transactivation by MDM2. Yeast were stably transfected with expression plasmids encoding p53, lex-VP16, MDM2 or the appropriate vector-only controls, as indicated. p53-responsive (bars a-c) or lexA-responsive (bars d-f) β-galactosidase reporter plasmids were used to assess the response.

Inset: Western blot analysis demonstrating MDM2 (90 kD) and p53 (53 kD) expression in representative yeast strains. The strain indicated by a plus was transfected with expression vector encoding full length MDM2 and p53, while the strain indicated by a minus was transfected only with the p53 expression vector.

Figure 6 shows the determination of MDM2 and p53 domains of interaction. Fig. 5A and Fig. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive β-galactosidase reporter). Yeast clones expressing β-galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined. β-galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. Fig. 6C. Random fragments of p53 were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Figure 7 shows protein expression from the yeast strains described in Figure 6. Western blot analysis was performed as described (Oliner, J.D., et al., *Nature 358*:80-83 (1992)), using 20 μg of protein per lane. The MDM2 and p53 codons contained in the fusion vectors are shown at the top of A and B, respectively. Fig. 7A. Upper panel probed with p53 Ab2 detecting p53; lower panel probed with anti-lexA polyclonal antibodies (lex Ab) detecting MDM2 fusion proteins of 30-50 kD. Fig. 7B. Upper panel probed with Lex Ab detecting the lexA-full length MDM2 fusion protein of 112 kD; lower panel probed with HA Ab (a monoclonal antibody directed against the hemagglutinin epitope tag, Berkeley Antibody) detecting p53 fusion proteins of approximately 25-30 kD.

Figure 8 shows the inhibition of the p53 activation domain by MDM2. Yeast were transfected with expression vectors encoding a lexA-p53 (p53 codons 1-73) fusion (bars a and b) or lexA alone (bar c). Strain b also expressed full length MDM2, and all strains contained the lexA-responsive β-galactosidase reporter plasmid. Inset: Upper panel probed with MDM2 polyclonal antibodies detecting full length MDM2 (90 kD); lower panel probed with lex Ab detecting the lex-p53 fusion protein of 40 kD.

Figure 9 shows a Western blot analysis using monoclonal antibodies to MDM2 or p53. Fifty μg of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis. The position of molecular weight markers, in kd, is given on the right.

Figure 10 demonstrates immunocytochemical analysis of OsA-CL and SW480 cells grown *in vitro*. Monoclonal antibody IF-2, specific for MDM2, and mAb 1801, specific for p53, were used. The exclusively nuclear localization of both proteins is evident, as is the higher expression of MDM2 protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for p53.

Figure 11 demonstrates MDM2 expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for MDM2. Tumors #3 and #10 showed nuclear expression of MDM2, while tumor #2 showed no staining.

DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that a gene exists which is amplified in some human tumors. The amplification of this gene, designated MDM2, is diagnostic of neoplasia or the potential therefor. Detecting the elevated expression of human MDM2-encoded products is also diagnostic of neoplasia or the potential for neoplastic transformation. Over a third of the sarcomas surveyed, including the most common bone and soft tissue forms, were found to have amplified hMDM2 sequences. Expression of hMDM2 was found to be correspondingly elevated in tumors with the gene amplification.

Other genetic alterations leading to elevated hMDM2 expression may be involved in tumorigenesis also, such as mutations in regulatory regions of the gene. Elevated expression of hMDM2 may also be involved in tumors other than sarcomas including but not limited to those in which p53 inactivation has been implicated. These include colorectal carcinoma, lung cancer and chronic myelogenous leukemia.

According to one embodiment of the invention, a method of diagnosing a neoplastic tissue in a human is provided. Tissue or body fluid is isolated from a human, and the copy number of human MDM2 genes is determined. Alternatively, expression levels of human MDM2 gene products can be determined. These include protein and mRNA.

Body fluids which may be tested include urine, serum, blood, feces, saliva, and the like. Tissues suspected of being neoplastic are desirably separated from normal appearing tissue for analysis. This can be done by paraffin or cryostat sectioning or flow cytometry, as is known in the art. Failure to separate neoplastic from non-neoplastic cells can confound the analysis. Adjacent non-neoplastic tissue or any normal tissue can be used to determine a base-line level of expression or copy number, against which the amount of hMDM2 gene or gene products can be compared.

The human MDM2 gene is considered to be amplified if the cell contains more than the normal copy number (2) of this gene per genome. The various techniques for detecting gene amplification are well known in the art. Gene amplification can be determined, for example, by Southern blot analysis, as described in Example 4, wherein cellular DNA from a human tissue is digested, separated, and transferred to a filter where it is hybridized with a probe containing complementary nucleic acids. Alternatively, quantitative polymerase chain reaction (PCR) employing primers can be used to determine gene amplification. Appropriate primers will bind to sequences that bracket human MDM2 coding sequences. Other techniques for determining gene copy number as are known in the art can be used without limitation.

The gene product which is measured may be either mRNA or protein. The term elevated expression means an increase in mRNA production or protein production over that which is normally produced by non-cancerous cells. Although amplification has been observed in human sarcomas, other genetic alterations leading to elevated expression of MDM2 may be present in these or other tumors. Other tumors include those of lung, breast, brain, colorectal, bladder, prostate, liver, skin, and stomach. These, too, are contemplated by the present invention. Non-cancerous cells for use in determining base-line expression levels can be obtained from cells surrounding a tumor, from other humans or from human cell lines. Any increase can have diagnostic value, but generally the mRNA or protein expression will be elevated at least about 3-fold, 5-fold, and in some cases up to about 100-fold over that found in non-cancerous cells. The particular technique employed for detecting mRNA or protein is not critical to the practice of the invention. Increased production of mRNA or protein may be detected, for example, using the techniques of Northern blot analysis or Western blot analysis, respectively, as described in Example 4 or other known techniques such as ELISA, immunoprecipitation, RIA and the like. These techniques are also well known to the skilled artisan.

According to another embodiment of the invention, nucleic acid probes or primers for the determining of human MDM2 gene amplification or elevated expression of mRNA are provided. The probe may comprise ribo- or deoxyribonucleic acids and may contain the entire human MDM2 coding sequence, a sequence complementary thereto, or fragments thereof. A probe may contain, for example, nucleotides 1-949, or 1-2372 as shown in Figure 1. Generally, probes or primers will contain at least about 14 contiguous nucleotides of the human sequence but may desirably contain about 40, 50 or 100 nucleotides. Probes are typically labelled with a fluorescent tag, a radioisotope, or the like to render them easily detectable. Preferably the probes will hybridize under stringent hybridization conditions. Under such conditions they will not hybridize to mouse MDM2. The probes of the invention are complementary to the human MDM2 gene. This means that they share 100% identity with the human sequence.

hMDM2 protein can be produced, according to the invention, substantially free of other human proteins. Provided with the DNA sequence, those of skill in the art can express the cDNA in a non-human cell. Lysates of such cells provide proteins substantially free of other human proteins. The lysates can be further purified, for example, by immunoprecipitation, co-precipitation with p53, or by affinity chromatography.

The antibodies of the invention are specifically reactive with hMDM2 protein. Preferably, they do not cross-react with MDM2 from other species. They can be polyclonal or monoclonal, and can be raised against native hMDM2 or a hMDM2 fusion protein or synthetic peptide. The antibodies are specifically immunoreactive with hMDM2 epitopes which are not present on other human proteins. Some antibodies are reactive with epitopes unique to human MDM2 and not present on the mouse homolog. The antibodies are useful in conventional analyses, such as Western blot analysis, ELISA, immunohistochemistry, and other immunological assays for the detection of proteins. Techniques for raising and purifying polyclonal antibodies are well known in the art, as are techniques for preparing monoclonal antibodies. Antibody binding can be determined by methods known in the art, such as use of an enzyme-labelled secondary antibody, staphylococcal protein A, and the like. Certain monoclonal antibodies of the invention have been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. These include IF2, and ED9, which have been granted accession nos. HB 11290, and HB 11291, respectively.

According to another embodiment of the invention, interference with the expression of MDM2 provides a therapeutic modality. The method can be applied in vivo, in vitro, or ex vivo. For example, expression may be down-regulated by administering triple-strand forming or antisense oligonucleotides which bind to the hMDM2 gene or mRNA, respectively, and prevent transcription or translation. The oligonucleotides may interact with unprocessed pre-mRNA or processed mRNA. Small molecules and peptides which specifically inhibit MDM2 expression can also be used.

Similarly, such molecules which inhibit the binding of MDM2 to p53 would be therapeutic by alleviating the sequestration of p53.

Such inhibitory molecules can be identified by screening for interference of the hMDM2/p53 interaction where one of the binding partners is bound to a solid support and the other partner is labeled. Antibodies specific for epitopes on hMDM2 or p53 which are involved in the binding interaction will interfere with such binding. Solid supports which may be used include any polymers which are known to bind proteins. The support may be in the form of a filter, column packing matrix, beads, and the like. Labeling of proteins can be accomplished according to any technique known in the art. Radiolabels, enzymatic labels, and fluorescent labels can be used advantageously. Alternatively, both hMDM2 and p53 may be in solution and bound molecules separated from unbound subsequently. Any separation technique known in the art may be employed, including immunoprecipitation or immunoaffinity separation with an antibody specific for the unlabeled binding partner.

It has been found that amino acid residues 13-41 of p53 (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and p53. However, additional residues on either the amino or carboxy terminal side of the peptide appear also to be required. Nine to 13 additional p53 residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from p53-regulated growth control in sarcomas, the use of p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53-regulated growth control.

Suitable p53-derived peptides for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

DNA encoding the MDM2-binding, p53-derived peptide, or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus,

or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, *inter alia* encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of peptide, the goal is to alleviate the sequestration of p53 by MDM2.

A cDNA molecule containing the coding sequence of hMDM2 can be used to produce probes and primers. In addition, it can be expressed in cultured cells, such as *E. coli*, to yield preparations of hMDM2 protein substantially free of other human proteins. The proteins produced can be purified, for example, with immunoaffinity techniques using the antibodies described above.

Kits are provided which contain the necessary reagents for determining gene copy number, such as probes or primers specific for the hMDM2 gene, as well as written instructions. The instructions can provide calibration curves to compare with the determined values. Kits are also provided to determine elevated expression of mRNA (*i.e.*, containing probes) or hMDM2 protein (*i.e.*, containing antibodies). Instructions will allow the tester to determine whether the expression levels are elevated. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to human p53. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

The following examples are provided to exemplify various aspects of the invention and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

To obtain human cDNA clones, a cDNA library was screened with a murine MDM2 (mMDM2) cDNA probe. A cDNA library was prepared by using polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 as a template for the production of random hexamer primed double stranded cDNA. Gubler and Hoffmann, 1983, *Gene 25*:263-268. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged, and plated as described by Elledge et al. (*Proc. Natl. Acad. Sci. USA, 88*:1731-1735, 1991). The library was screened initially with a P-labelled (Kinzler, K.W., et al., *Nucl. Acids Res. 17*:3645-3653 (1989), Feinberg and Vogelstein, 1983, *Anal. Biochem. 132*.6-13) mMDM2 cDNA probe (nucleotides 259 to 1508 (Fakharzadeh et al., 1991, *EMBO J. 10*:1565-1569)) and then rescreened with an hMDM2 cDNA clone containing nucleotides 40 to 702.

Twelve clones were obtained, and one of the clones was used to obtain thirteen additional clones by re-screening the same library. In total, twenty-five clones were obtained, partially or totally sequenced, and mapped. Sequence analysis of the twenty-five clones revealed several cDNA forms indicative of alternative splicing. The sequence shown in Figure 1 is representative of the most abundant class and was assembled from three clones: c14-2 (nucleotides 1-949), c89 (nucleotides 467-1737), and c33 (nucleotides 390-2372). The 3' end of the untranslated region has not yet been cloned in mouse or human. The 5' end is likely to be at or near nucleotide 1. There was an open reading frame extending from the 5' end of the human cDNA sequence to nucleotide 1784. Although the signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between hMDM2 and mMDM2 fell off dramatically upstream of nucleotide 312. This lack of conservation in an otherwise highly conserved protein suggested that the sequences upstream of the divergence may not code for protein. Second, an anchored polymerase chain reaction (PCR) approach was employed in an

effort to acquire additional upstream cDNA sequence. Ochman et al., 1985, In: *PCR Technology: Principles and Applications for DNA Amplification* (Erlich, ed.) pp. 105-111 (Stockton, New York). The 5' ends of the PCR derived clones were very similar (within 3 bp) to the 5' ends of clones obtained from the cDNA library, suggesting that the 5' end of the hMDM2 sequence shown in Figure 1 may represent the 5' end of the transcript. Third, *in vitro* translation of the sequence shown in Figure 1, beginning with the methionine encoded by the nucleotide 312 ATG, generated a protein similar in size to that observed in human cells.

In Figure 1, hMDM2 cDNA sequence, hMDM2 and mMDM2 nucleotide and amino acid sequences are compared. The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5' and 3' boundaries of the previously published mMDM2 cDNA. Fakharzadeh et al., 1991, *EMBO J. 10*:1565-1569. Dashes indicate insertions. The mouse and human amino acid sequences are compared from the putative translation start site at nucleotide 312 through the conserved stop codon at nucleotide 1784.

Comparison of the human and mouse MDM2 coding regions revealed significant conservation at the nucleotide (80.3%) and amino acid (80.4%) levels. Although hMDM2 and mMDM2 bore little similarity to other genes recorded in current databases, the two proteins shared several motifs. These included a basic nuclear localization signal (Tanaka, 1990, *FEBS Letters 271*:41-46) at codons 181 to 185, several casein kinase II serine phosphorylation sites (Pinna, 1990, *Biochem. et. Biophys. Acta. 1054*:267-284) at codons 166 to 169, 192 to 195, 269 to 272, and 290 to 293, an acidic activation domain (Ptashne, 1988, *Nature 355*:683-689) at codons 223 to 274, and two metal binding sites (Harrison, 1991, *Nature 353*:715) at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA binding domains. The protein kinase A domain noted in mMDM2 (Fakharzadeh et al., 1991, *EMBO J. 10*:1565-1569) was not conserved in hMDM2.

Example 2

To determine whether the hMDM2 protein could bind to human p53 protein *in vitro*, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Figure 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, *Nucleic Acid Research 15*:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, *Nature Genetics*, in press) and MCC (Kinzler et al., 1991, *Science 251*:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Although the predicted size of the protein generated from the construct was only 55.2 kd (extending from the methionine at nucleotide 312 to nucleotide 1784), *in vitro* translated protein migrated at approximately 95 kilodaltons.

Ten μl of lysate containing the three proteins (hMDM2, p53 and MCC), alone or mixed in pairs, were incubated at 37°C for 15 minutes. One microgram (10 μl) of p53 Ab1 (monoclonal antibody specific for the C-terminus of p53) or Ab2 (monoclonal antibody specific for the N-terminus of p53) (Oncogene Science), or 5 μl of rabbit serum containing MDM2 Ab (polyclonal rabbit anti-hMDM2 antibodies) or preimmune rabbit serum (obtained from the rabbit which produced the hMDM2 Ab), were added as indicated. The polyclonal rabbit antibodies were raised against an *E. coli*-produced hMDM2-glutathione S-transferase fusion protein containing nucleotides 390 to 816 of the hMDM2 cDNA. Ninety μl of RIPA buffer (10 mM tris [pH 7.5], 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer, or Binding Buffer (El-Deriy et al., 1992, *Nature Genetics*, in press) were then added and the mixtures allowed to incubate at 4°C for 2 hours.

Two milligrams of protein A sepharose were added to each tube, and the tubes were rotated end-over-end at 4°C for 1 hour. After pelleting and washing, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and the dried gels autoradiographed for 10 to 60 minutes in the presence of Enhance (New England Nuclear).

Figure 2 shows the co-precipitation of hMDM2 and p53. The three buffers produced similar results, although the co-precipitation was less efficient in SNNTE buffer containing 0.5 M NaCl (Figure 2, lanes 5 and 8) than in Binding Buffer containing 0.1 M NaCl (Figure 2 lanes 6 and 9).

In vitro translated hMDM2, p53 and MCC proteins were mixed as indicated above and incubated with p53 Ab1, p53 Ab2, hMDM2 Ab, or preimmune serum. Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. The bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (Figure 2, lanes 2 and 3). However, when *in vitro* translated human p53 was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association *in vitro* (Figure 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, *Science 251*:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (Figure 2, lanes 8 and 9). When an *in vitro* translated mutant form of p53 (175^{his}) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

In the converse of the experiments described above, the anti-hMDM2 antibodies immunoprecipitated p53 when mixed with hMDM2 protein (Figure 2, lane 15) but failed to precipitate p53 alone (Figure 5, lane 13). Preimmune rabbit serum failed to precipitate either hMDM2 or p53 (Figure 2, lane 16).

Example 3

In order to ascertain the chromosomal localization of hMDM2, somatic cell hybrids were screened with an hMDM2 cDNA probe. A human-hamster hybrid containing only human chromosome 12 was found to hybridize to the probe. Screening of hybrids containing portions of chromosome 12 (Turc-Carel et al., 1986, *Cancer Genet. Cytogenet. 23*:291-299) with the same probe narrowed the localization to chromosome 12q12-14.

Example 4

Previous studies have shown that this region of chromosome 12 is often aberrant in human sarcomas. Mandahl et al., 1987, Genes Chromosomes & Cancer 1:9-14; Turc-Carel et al., 1986, *Cancer Genet. Cytogenet. 23*:291-299; Meltzer et al., 1991, *Cell Growth & Differentiation 2*:495-501. To evaluate the possibility that hMDM2 was genetically altered in such cancers, Southern blot analysis was performed.

Figure 3 shows examples of the amplification of the hMDM2 gene in sarcomas. Cellular DNA (5 μg) was digested with *Eco*RI, separated by agarose gel electrophoresis, and transferred to nylon as described by Reed and Mann (*Nucl. Acids Res., 1985, 13*:7207-7215). The cellular DNA was derived from five primary sarcomas (lanes 1-4, 6) and one sarcoma cell line (OsA-C1, lane 5). The filters were then hybridized with an hMDM2 cDNA fragment probe nucleotide 1-949 (see Figure 1), or to a control probe which identifies fragments of similar size (DCC gene, 1.65 cDNA fragment). Fearon, 1989, *Science 247*:49-56. Hybridization was performed as described by Vogelstein et al. (*Cancer Research, 1987, 47*:4806-4813). A striking amplification of hMDM2 sequences was observed in several of these tumors. (See Figure 3, lanes 2, 3 and 5). Of 47 sarcomas analyzed, 17 exhibited hMDM2 amplification ranging from 5 to 50 fold. These tumors included 7 to 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas (MFH), 3 of 11 osteosarcomas, and 0 and 1 rhabdomyosarcomas. Five benign soft tissue tumors (lipomas) and twenty-seven carcinomas (colorectal or gastric) were also tested by Southern blot analysis and no amplification was observed.

Example 5

This example illustrates that gene amplification is associated with increased expression.

Figure 4A illustrates hMDM2 expression as demonstrated by Northern blot analysis. Because of RNA degradation in the primary sarcomas, only the cell lines could be productively analyzed by Northern blot. RNA was separated by electrophoresis in a MOPS-formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were performed as described by Kinzler et al. (*Nature 332*:371-374, 1988). The RNA was hybridized to the hMDM2 fragment described in Figure 3. Ten μg of total RNA derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains 10 μg of polyadenylated CaCo-2 RNA. RNA sizes are shown in kb. In the one available sarcoma cell line with hMDM2 amplification, a single transcript of approximately 5.5 kb was observed (Figure 4A, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Figure 4A, lane 2) or in a carcinoma cell line (Figure 4A, lane 3). When purified mRNA (rather than total RNA) from the carcinoma cell line was used for analysis, an hMDM2 transcript of 5.5 kb could also be observed (Figure 4A, lane 4).

Figure 4B illustrates hMDM2 expression as demonstrated by Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2).

Figure 4C illustrates hMDM2 expression as demonstrated by Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with hMDM2 amplifications, and lanes 4 and 5 contain protein from sarcomas without hMDM2 amplification.

Western blots using affinity purified MDM2 Ab were performed with 50 μg protein per lane as described by Kinzler et al. (*Mol. Cell. Biol., 1990, 10*:634-642), except that the membranes were blocked in 10% nonfat dried milk and 10% goat serum,

and secondary antibodies were coupled to horseradish peroxidase, permitting chemiluminescent detection (Amersham ECL). MDM2 Ab was affinity purified with a pATH-hMDM2 fusion protein using methods described in Kinzler et al. (*Mol. Cell. Biol. 10*:634-642, 1990). Non-specifically reactive proteins of about 75-85, 105-120 and 170-200 kd were observed in all lanes, irrespective of hMDM2 amplification status. hMDM2 proteins, of about 90-97 kd, were observed only in the hMDM2-amplified tumors. Protein marker sizes are shown in kd.

A protein of approximately 97 kilodaltons was expressed at high levels in the sarcoma cell line with hMDM2 amplification (Figure 4B, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Figure 4B, lanes 1, 2 and 4). Five primary sarcomas were also examined by Western blot analysis. Three primary sarcomas with amplification expressed the same size protein as that observed in the sarcoma cell line (Figure 4C, lanes 1-3), while no protein was observed in the two sarcomas without amplification (Figure 4C, lanes 4 and 5).

Expression of the hMDM2 RNA in the sarcoma with amplification was estimated to be at least 30 fold higher than that in the other lines examined. This was consistent with the results of Western blot analysis.

The above examples demonstrate that hMDM2 binds to p53 *in vitro* and is genetically altered (*i.e.*, amplified) in a significant fraction of sarcomas, including MFH, liposarcomas, and osteosarcomas. These are the most common sarcomas of soft tissue and bone. Weiss and Enzinger, 1978, *Cancer 41*:2250-2266; Malawer et al., 1985, In: *Cancer: Principles and Practice of Oncology*, DeVita et al., Eds., pp. 1293-1342 (Lippincott, Philadelphia).

Human MDM2 amplification is useful for understanding the pathogenesis of these often lethal cancers.

MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as SV40 T-antigen, adenovirus E1B, and HPV E6. Lane and Bechimol, 1990, *Genes and Development 4*:1-8; Werness et al., 1990, *Science*

248:76. Consistent with this hypothesis, no sarcomas with hMDM2 amplification had any of the p53 gene mutations that occur commonly in other tumors. hMDM2 amplification provides a parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. The finding that expression of hMDM2 is correspondingly elevated in tumors with amplification of the gene are consistent with the finding that MDM2 binds to p53, and with the hypothesis that overexpression of MDM2 in sarcomas allows escape from p53 regulated growth control. This mechanism of tumorigenesis has striking parallels to that previously observed for virally induced tumors (Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, *Science 248*:76), in which viral oncogene products bind to and functionally inactivate p53.

Example 6

This example demonstrates that MDM2 expression inhibits p53-mediated transactivation.

To determine if MDM2 could influence the ability of p53 to activate transcription, expression vectors coding for the two proteins were stably transfected into yeast along with a p53-responsive reporter construct. The reporter consisted of a β-galactosidase gene under the transcriptional control of a minimal promoter and a multimerized human DNA sequence which strongly bound p53 *in vitro* (Kern, S.E., et al., *Science 256*:827-830 (1992). Reporter expression was completely dependent on p53 in this assay (Figure 5, compare bars a and c). MDM2 expression was found to inhibit p53-mediated transactivation of this reporter 16-fold relative to isogeneic yeast lacking MDM2 expression (Figure 5, compare bars a and b). Western blot analysis confirmed that p53 (53 kD) was expressed equivalently in strains with and without MDM2 (90 kD) (Figure 1, inset).

METHODS. The MDM2 expression plasmid, pPGK-MDM2, was constructed by inserting the full length MDM2 cDNA (Oliner, J.D., et al., *Nature 358*:80-83 (1992)) into pPGK (Poon, D. *et al., Mol. and Cell.*

Biol. 1111:4809-4821 (1991)), immediately downstream of the phosphoglycerate kinase constitutive promoter. Galactose-inducible p53 (pRS314SN, Nigro, J.M., et al., *Mol. and Cell. Biol. 12*:1357-1365 (1992)), lexA-VP16 (YVLexA, Dalton, S., et al., *Cell 68*:597-612 (1992)), and lexA (YLexA, YVLexA minus VP16) plasmids were used as indicated. The reporters were PG16-*lacZ* (Kern, S.E. *et al., Science 256*:827-830 (1992)) (p53-responsive) and pJK103 (Kamens, J., et al., *Mol. Cell. Biol. 10*:2840-2847 (1990)) (lexA-responsive). *S. cerevisiae* strain pEGY48 was transformed as described (Kinzler, K.W. et al., *Nucl. Acids Res. 17*:3645-3653 (1989)). Yeast strains represented by bars a-c were grown at 30°C to mid-log phase in selective liquid medium containing 2% raffinose as the carbon source, induced for 30 minutes by the addition of 2% galactose, harvested, and lysed as described (Kern, S.E. *et al., Science 256*:827-830 (1992)). The strains represented by bars d-f were treated similarly, except that the cells were induced in galactose for 4 hours to obtain measurable levels of β-galactosidase. β-galactosidase activities shown represent the mean of three to five experimental values (error bars indicate s.e.m.). Protein concentrations were determined by a Coomassie blue-based (bio-Rad) assay. The β-galactosidase assays were performed with AMPGD chemiluminescent substrate and Emerald enhancer (Tropix) according to the manufacturer's instructions. β-galactosidase activities of bars b and c are shown relative to that of bar A; β-galactosidase activities of bars e and f are shown relative to that of bar d. Western blots were performed as described (Oliner, J.D., et al., *Nature 358*:80-83 (1992)), using p53 Ab1801 (lower panel, Oncogene Science) or MDM2 polyclonal antibodies (Oliner, J.D., et al., *Nature 358*:80-83 (1992)) (upper panel).

To ensure that this inhibition was not simply a general transcriptional down regulation mediated by the expression of the foreign MDM2 gene, a yeast strain was created that contained a different transcriptional activator (lexA-VP16, consisting of the lexA DNA binding domain fused to the VP16 acidic activation domain), a similar reporter (with a lexA-responsive site upstream of a β-galactosidase gene), and the same MDM2 expression vector. The results shown in Figure 1 (bars d & e) demonstrate that lexA-VP16 transactivation was unaffected by the presence of MDM2. Furthermore, MDM2 expression had no apparent effect on the growth rate of the cells.

Example 7

This example demonstrates the domains of p53 and MDM2 which interact with each other.

To gain insight into the mechanism of the MDM2-mediated p53 inhibition, the domains of MDM2 and p53 responsible for binding to one another were mapped. The yeast system used to detect protein-protein binding takes advantage of the modular nature of transcription factor domains (Keegan, L., et al., *Science 231*:699-704 (1986); Chien, C.-T., *Proc. Natl. Acad. Sci. U.S.A. 88*:9578-9582 (1991); Brent, R., et al., *Cell 43*:729-731 (1985); Ma, J., et al., *Cell 55*:4430446 (1988). Generically, if protein 1 (fused to a sequence-specific DNA binding domain) is capable of binding to protein 2 (fused to a transcriptional activation domain), then co-expression of both fusion proteins will result in transcriptional activation of a suitable reporter. In our experiments, the lexA DNA binding domain (amino acids 2-202) and the B42 acidic activation domain (AAD) were used in the fusion constructs. The reporter (Kamens, J., et al., *Mol. Cell. Biol. 10*:2840-2847 (1990); contained a lexA-responsive site upstream of a β-galactosidase gene. As an initial control experiment, full length MDM2 was inserted into the lexA fusion vector, and full length p53, supplying its intrinsic activation domain was inserted into a non-fusion vector. The combination resulted in the activation of the lexA-responsive reporter, while the same expression constructs lacking either the MDM2 or p53 cDNA inserts failed to activate β-galactosidase (Table I, strains 1, 2, and 3). Thus, activation was dependent upon MDM2-p53 binding.

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA fragments encoding MDM2 or p53 were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K.W., et al., *Nucl. Acids Res. 17*:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D.M., et al., *Nature 350*:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive β-galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53-independent clones are diagrammed in Fig. 6A. The MDM2 sequences of the remaining 15 p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of Fig. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-terminal to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three p53 sequences shown in Fig. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated fragments.

The resultant yeast colonies were examined for β-galactosidase activity *in situ*. Of approximately 5000 clones containing MDM2 fragments fused to the lexA DNA

binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of β-galactosidase (about 5-fold less than the other fifteen clones) and β-galactosidase expression was independent of p53 expression (Figure 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable β-galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino terminal region of MDM2 (Figure 6B). The β-galactosidase activity of these clones was dependent on p53 co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length p53 contained MDM2 codons 1 to 118 (Figure 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated fragments of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

In a converse set of experiments, yeast clones containing fragments of p53 fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (Figure 2C). The minimal overlap between these three fragments contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the fragments required 9-12 amino acids on either side of codons 13-41 for activity (Figure 6C). To further test the idea that the amino terminal region of p53 was required for MDM2 binding, we generated an additional yeast strain expressing

the lexA-DNA binding domain fused to p53 codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-terminus of p53 were required for the interaction.

TABLE I

STRAIN NUMBER	p53 CONSTRUCT	MDM2 CONSTRUCT	ACTIVATION
1	p53[a]	Vector[b]	-
2	p53[a]	lexA-MDM2[b]	+
3	Vector[a]	lexA-MDM2[b]	-
4	p53[a]	lexA-MDM2 (1-118)[b]	+
5	Vector[a]	lexA-MDM2 (1-118)[b]	-
6	B42-p53 (1-41)[c]	lexA-MDM2[b]	+
7	B42-p53 (1-41)[c]	Vector[b]	-
8	lexA-p53 (74-393)[b]	B42-MDM2[c]	-
9	p53 (1-137)[a]	lexA-MDM2[b]	-

The MDM2 and p53 proteins expressed in each strain, along with the relevant reporters, are indicated. Numbers in parentheses refer to the MDM2 or p53 amino acids encoded (absence of parentheses indicated full length protein, that is, MDM2 amino acids 1 to 491 or p53 amino acids 1 to 393). The lexA-responsive β-galactosidase reporter plasmid (pJK103, Kamens, J., et al., *Mol. Cell. Biol. 10*:2840-2847 (1990)) was present in all strains.

[a]pRS314 vector (Nigro, J.M., et al., *Mol. and Cell. Biol. 12*:1357-1365 (1992).

[b]plex(1-202)+PL vector, containing lexA DNA binding domain fused to insert (Ruden, D.M., et al., *Nature 350*:250-252 (1991).

[c]pJG4-5 vector, containing B42 activation domain fused to insert.

[d](+) indicates that colonies turned blue following 24 hours of incubation on X-gal-containing selective medium, while (-) indicates that colonies remained white following 72 hours of incubation.

Sequence analysis showed that all p53 and MDM2 fragments noted in Figure 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in Figure 6 expressed the relevant proteins at similar levels, as shown by Western blotting (Figure 7).

The most striking results of these mapping experiments was that the region of p53 required to bind MDM2 was almost identical to the previously identified acidic activation domain of p53 (amino acids 20-42) (Unger, T., et al., *EMBO J. 11*:1383-1390 (1992); Miller, C.W., et al., *Proc. Am. Assoc. Cancer Res. 33*:386 (1992). This suggested that MDM2 inhibits p53-mediated transcriptional activation by "concealing" the activation domain of p53 from the transcriptional machinery. If this were true, the p53 activation domain, in isolation from the rest of the p53 protein, should still be inhibitable by full length MDM2. To test this hypothesis, we produced a hybrid protein containing the p53 activation domain (codons 1-73) fused to the lexA-DNA binding domain. This construct exhibited strong transcriptional activation of a lexA-responsive reporter (Figure 8), as predicted from previous experiments in which the p53 activation domain was fused to another DNA binding domain (Fields, S., et al., *Science 249*:1046-1049 (1990); Raycroft, L., et al., *Science 249*:1049-1051 (1990)). The lexA-p53 DNA construct was stably expressed in yeast along with the full length MDM2 expression vector (or the vector alone). MDM2 expression resulted in a five-fold decrease in reporter activity, demonstrating that MDM2 can specifically inhibit the function of the p53 activation domain regardless of the adjacent protein sequences tethering p53 to DNA (Figure 8).

METHODS. Strains were grown to mid-log phase in 2% dextrose before induction of p53 expression for 2 hours by the addition of 2% galactose. The lex-p53 construct was identical to lex-VP16 (YVlexA, Dalton, S., et al., *Cell 68*:597-612 (1992)) except that VP16 sequences were replaced by p53 sequences encoding amino acids 1 to 73.

The results obtained in the experiments discussed herein raise an interesting paradox. If MDM2 binds to (Figure 6) and conceals (Figure 8) the p53 activation

domain from the transcriptional machinery, how could the lexA-MDM2-p53 complex activate transcription from the lexA-responsive reporter (Table I, strain 2)? Because the only functional activation domain in the lexA-MDM2-p53 complex of strain 2 is expected to be contributed by p53, one might predict that it would be concealed by binding to MDM2 and thereby fail to activate. A potential resolution of this paradox is afforded by knowledge that p53 exists as a homotetramer (Stenger, J.E., et al., *Mol. Carcinogenesis* 5:102-106 (1992); Sturzbecher, H.W. *et al.*, *Oncogene* 7:1513-1523 (1992). Thus the activation noted in the lexA-MDM2-p53 complex could be due to the presence of four individual activation domains contributed by the p53 tetramer, not all of which were concealed by MDM2. As a direct test of this issue, the domain of p53 required for homo-oligomerization (Stenger, J.E., et al., *Mol. Carcinogenesis* 5:102-106 (1992); Sturzbecher, H.W. *et al.*, *Oncogene* 7:1513-1523 (1992) (the C-terminus) was removed from the p53 expression construct, so that it consisted of only codons 1-137. This truncated p53 polypeptide retained the entire activation domain (as shown in Figure 8, bar a) and the entire domain required for interaction with MDM2 (Table I, strain 6). Yet, when allowed to interact with lexA-MDM2, no transactivation of the lexA-responsive reporter was observed (Table I, strain 9). Because p53 did not inhibit lexA-MDM2 binding to the lexA reporter (Table I, strain 2), the result of strain 9 is likely to be due to a direct inhibition of the isolated p53 activation domain by MDM2.

Example 8

This example illustrates the production and characterization of antibodies specific for MDM2 epitopes.

The antigen preparations used to intraperitoneally immunize female (BALB/c X C57BL/6)F1 mice comprised bacterially expressed, glutathione-column purified glutathione-S-transferase-MDM2 (GST-MDM2) fusion protein. (One preparation was further purified on a polyacrylamide gel and electroeluted.) The fusion protein contains a 16 kD amino terminal portion of human MDM2 protein (amino acids 27 to

168). For immunization, the fusion protein was mixed with Ribi adjuvant (Ribi Immunochem Research, Inc.).

Two mice were sacrificed and their spleen cells fused to SP2/0s myeloma cells (McKenzie, et al., *Oncogene*, *4*:543-548, 1989). Resulting hybridomas were screened by ELISA on trpE-MDM2 fusion protein-coated microtiter wells. The trpE-MDM2 fusion protein contains the same portion of MDM2 as the GST-MDM2 fusion protein. Antigen was coated at a concentration of 1 μg/ml.

A second fusion was performed as described except hybridomas were screened on electroeluted, glutathione purified GST-MDM2. Positive hybridomas from both fusions were expanded and single cell subcloned. Subclones were tested by Western Blot for specificity to the 55 kD trpE-MDM2 and the 43 kD GST-MDM2 fusion proteins.

Two Western Blot positive subclones (1F2 and JG3) were put into mice for ascites generation. The resulting ascites were protein A purified. Both purified monoclonal antibodies tested positive by Western Blot and immunoprecipitation for the 90 kD migrating MDM2 protein present in a human osteosarcoma cell line (OsA-CL), which overexpresses MDM2, and negative in the HOS osteosarcoma, which does not overexpress MDM2.

ED9 was protein G-purified from ascites and found to be specific in cryostat immunohistochemistry for MDM2 in osteosarcoma cells, as was IF2.

Example 9

This example demonstrates the expression and detection of MDM2 at the cellular level.

To evaluate MDM2 expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. (See example 8.) Of several antibodies tested, mAb IF-2 was the most useful, as it detected MDM2 in several assays. For initial testing, we compared proteins derived

from OsA-CL, a sarcoma cell line with MDM2 amplification but without p53 mutation (Table II) and proteins from SW480, a colorectal cancer cell line with p53 mutation (Barak et al., *EMBO 12*:461-468 (1993)) but without MDM2 amplification (data not shown). Figure 9 shows that the mAb IF-2 detected an intense 90 kd band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense 90 kd band in SW480 extracts. We could not distinguish whether the low molecular weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in MDM2 gene copy number in these two lines. Conversely, the 53 kd signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL consistent with the presence of a mutated p53 in SW480 (Fig. 9).

Cells grown on cover slips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 10). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (Barak et al., *EMBO 12*:461-468 (1993)) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186. Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 10), with the relative intensities consistent with the Western blot results (Fig. 9).

The IF-2 mAb was then used (at 5 μg/ml) to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors #3 and #10) stained strongly (Fig. 11). Both of these tumors contained MDM2 gene amplification (Table II). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 11).

TABLE II

TUMOR #	TUMOR ID	TYPE[a]	MDM2 AMPLIFICATION[b]	P53 ALTERATION[c]	OVER-EXPRESSION[d]
1	M-2	MFH	ABSENT	DELETION/ REARRANGEMENT	NONE
2	M-5	MFH	ABSENT	CGC-CUC MUTATION; Arg(158)-His	p53
3	M-7	MFH	PRESENT	NONE OBSERVED	MDM2
4	M-8	MFH	ABSENT	DELETION	NONE
5	M-14	MFH	ABSENT	NONE OBSERVED	N.T.
6	M-15	MFH	ABSENT	DELETION	N.T.
7	M-16	MFH	ABSENT	NONE OBSERVED	NONE
8	M-17	MFH	ABSENT	NONE OBSERVED	N.T.
9	M-18	MFH	ABSENT	OVEREXPRESSED	p53
10	M-20	MFH	PRESENT	NONE OBSERVED	MDM2
11	L-5	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
12	L-7	LIPOSARCOMA	ABSENT	AAC-AGC MUTATION; Asn(239)-Ser	N.T.
13	L-9	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.

TABLE II (Cont.)

TUMOR #	TUMOR ID	TYPE[a]	MDM2 AMPLIFICATION[b]	P53 MUTATION[c]	OVER-EXPRESSION[d]
14	L-11	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
15	KL5B	LIPOSARCOMA	ABSENT	CAG-UAG MUTATION; Gln(144)-Stop	N.T.
16	KL7	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
17	KL10	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
18	KL11	LIPOSARCOMA	ABSENT	GGT-GAT MUTATION; EXON 5 SPLICE DONOR SITE	N.T.
19	KL12	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
20	KL28	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
21	KL30	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
22	S189	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
23	S131B	LIPOSARCOMA	ABSENT	NONE OBSERVED	N.T.
24	OSA-CL	MFH	PRESENT	NONE OBSERVED	MDM2

[a] MFH= malignant fibrous histiocytoma

[b] as assessed by Southern blot

[c] as assessed by Southern blot, sequencing of exons 5-8, or immunohistochemical analysis

[d] as assessed by immunohistochemical analysis; N.T. = not tested

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BURRELL, MARILEE
HILL, DAVID E.
KINZLER, KENNETH W.
VOGELSTEIN, BERT

(ii) TITLE OF INVENTION: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: BANNER, BIRCH, MCKIE AND BECKETT
(B) STREET: 1001 G STREET, N.W.
(C) CITY: WASHINGTON
(D) STATE: D.C.
(E) COUNTRY: USA
(F) ZIP: 20001

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 07-APR-1993

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: KAGAN, SARAH A.

(B) REGISTRATION NUMBER: 32,141

(C) REFERENCE/DOCKET NUMBER: 01107.42798

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 202-508-9100

(B) TELEFAX: 202-508-9299

(C) TELEX: 197430 BBMB UT

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 64 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 17q

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln
1 5 10 15

Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu
20 25 30

Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp
35 40 45

Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro
50 55 60

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2372 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(H) CELL LINE: CaCo-2

(viii) POSITION IN GENOME:

(B) MAP POSITION: 12q12-14

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 312..1784

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGGCC CTGTGTGTCG GAAAGATGGA 60

GCAAGAAGCC GAGCCCGAGG GGCGGCCGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG 120

CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG 180

GAGAGTGGAA TGATCCCCGA GGCCCAGGGC GTCGTGCTTC CGCAGTAGTC AGTCCCCGTG 240

AAGGAAACTG GGGAGTCTTG AGGGACCCCC GACTCCAAGC GCGAAAACCC CGGATGGTGA 300

GGAGCAGGCA A ATG TGC AAT ACC AAC ATG TCT GTA CCT ACT GAT GGT GCT 350
Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala
1 5 10

GTA ACC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACC CTG GTT AGA 398
Val Thr Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg
15 20 25

CCA AAG CCA TTG CTT TTG AAG TTA TTA AAG TCT GTT GGT GCA CAA AAA 446
Pro Lys Pro Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Lys
30 35 40 45

GAC ACT TAT ACT ATG AAA GAG GTT CTT TTT TAT CTT GGC CAG TAT ATT 494
Asp Thr Tyr Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile
50 55 60

ATG ACT AAA CGA TTA TAT GAT GAG AAG CAA CAA CAT ATT GTA TAT TGT 542
Met Thr Lys Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys
65 70 75

TCA AAT GAT CTT CTA GGA GAT TTG TTT GGC GTG CCA AGC TTC TCT GTG 590
Ser Asn Asp Leu Leu Gly Asp Leu Phe Gly Val Pro Ser Phe Ser Val
80 85 90

```
AAA GAG CAC AGG AAA ATA TAT ACC ATG ATC TAC AGG AAC TTG GTA GTA      638
Lys Glu His Arg Lys Ile Tyr Thr Met Ile Tyr Arg Asn Leu Val Val
     95                  100                 105

GTC AAT CAG CAG GAA TCA TCG GAC TCA GGT ACA TCT GTG AGT GAG AAC      686
Val Asn Gln Gln Glu Ser Ser Asp Ser Gly Thr Ser Val Ser Glu Asn
110                 115                 120                 125

AGG TGT CAC CTT GAA GGT GGG AGT GAT CAA AAG GAC CTT GTA CAA GAG      734
Arg Cys His Leu Glu Gly Gly Ser Asp Gln Lys Asp Leu Val Gln Glu
                130                 135                 140

CTT CAG GAA GAG AAA CCT TCA TCT TCA CAT TTG GTT TCT AGA CCA TCT      782
Leu Gln Glu Glu Lys Pro Ser Ser Ser His Leu Val Ser Arg Pro Ser
            145                 150                 155

ACC TCA TCT AGA AGG AGA GCA ATT AGT GAG ACA GAA GAA AAT TCA GAT      830
Thr Ser Ser Arg Arg Arg Ala Ile Ser Glu Thr Glu Glu Asn Ser Asp
        160                 165                 170

GAA TTA TCT GGT GAA CGA CAA AGA AAA CGC CAC AAA TCT GAT AGT ATT      878
Glu Leu Ser Gly Glu Arg Gln Arg Lys Arg His Lys Ser Asp Ser Ile
    175                 180                 185

TCC CTT TCC TTT GAT GAA AGC CTG GCT CTG TGT GTA ATA AGG GAG ATA      926
Ser Leu Ser Phe Asp Glu Ser Leu Ala Leu Cys Val Ile Arg Glu Ile
190                 195                 200                 205
```

TGT TGT GAA AGA AGC AGT AGC AGT GAA TCT ACA GGG ACG CCA TCG AAT 974
Cys Cys Glu Arg Ser Ser Ser Ser Glu Ser Thr Gly Thr Pro Ser Asn
210 215 220

CCG GAT CTT GAT GCT GGT GTA AGT GAA CAT TCA GGT GAT TGG TTG GAT 1022
Pro Asp Leu Asp Ala Gly Val Ser Glu His Ser Gly Asp Trp Leu Asp
225 230 235

CAG GAT TCA GTT TCA GAT CAG TTT AGT GTA GAA TTT GAA GTT GAA TCT 1070
Gln Asp Ser Val Ser Asp Gln Phe Ser Val Glu Phe Glu Val Glu Ser
240 245 250

CTC GAC TCA GAA GAT TAT AGC CTT AGT GAA GAA GGA CAA GAA CTC TCA 1118
Leu Asp Ser Glu Asp Tyr Ser Leu Ser Glu Glu Gly Gln Glu Leu Ser
255 260 265

GAT GAA GAT GAT GAG GTA TAT CAA GTT ACT GTG TAT CAG GCA GGG GAG 1166
Asp Glu Asp Asp Glu Val Tyr Gln Val Thr Val Tyr Gln Ala Gly Glu
270 275 280 285

AGT GAT ACA GAT TCA TTT GAA GAA GAT CCT GAA ATT TCC TTA GCT GAC 1214
Ser Asp Thr Asp Ser Phe Glu Glu Asp Pro Glu Ile Ser Leu Ala Asp
290 295 300

TAT TGG AAA TGC ACT TCA TGC AAT GAA ATG AAT CCC CCC CTT CCA TCA 1262
Tyr Trp Lys Cys Thr Ser Cys Asn Glu Met Asn Pro Pro Leu Pro Ser
305 310 315

CAT TGC AAC AGA TGT TGG GCC CTT CGT GAG AAT TGG CTT CCT GAA GAT 1310
His Cys Asn Arg Cys Trp Ala Leu Arg Glu Asn Trp Leu Pro Glu Asp
320 325 330

AAA GGG AAA GAT AAA GGG GAA ATC TCT GAG AAA GCC AAA CTG GAA AAC 1358
Lys Gly Lys Asp Lys Gly Glu Ile Ser Glu Lys Ala Lys Leu Glu Asn
335 340 345

TCA ACA CAA GCT GAA GAG GGC TTT GAT GTT CCT GAT TGT AAA AAA ACT 1406
Ser Thr Gln Ala Glu Glu Gly Phe Asp Val Pro Asp Cys Lys Lys Thr
350 355 360 365

ATA GTG AAT GAT TCC AGA GAG TCA TGT GTT GAG GAA AAT GAT GAT AAA 1454
Ile Val Asn Asp Ser Arg Glu Ser Cys Val Glu Glu Asn Asp Asp Lys
370 375 380

ATT ACA CAA GCT TCA CAA TCA CAA GAA AGT GAA GAC TAT TCT CAG CCA 1502
Ile Thr Gln Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro
385 390 395

TCA ACT TCT AGT AGC ATT ATT TAT AGC AGC CAA GAA GAT GTG AAA GAG 1550
Ser Thr Ser Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu
400 405 410

TTT GAA AGG GAA GAA ACC CAA GAC AAA GAA GAG AGT GTG GAA TCT AGT 1598
Phe Glu Arg Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser
415 420 425

```
TTG CCC CTT AAT GCC ATT GAA CCT TGT GTG ATT TGT CAA GGT CGA CCT      1646
Leu Pro Leu Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro
430                 435                 440                 445

AAA AAT GGT TGC ATT GTC CAT GGC AAA ACA GGA CAT CTT ATG GCC TGC      1694
Lys Asn Gly Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys
            450                 455                 460

TTT ACA TGT GCA AAG AAG CTA AAG AAA AGG AAT AAG CCC TGC CCA GTA      1742
Phe Thr Cys Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val
        465                 470                 475

TGT AGA CAA CCA ATT CAA ATG ATT GTG CTA ACT TAT TTC CCC              1784
Cys Arg Gln Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro
    480                 485                 490

TAGTTGACCT GTCTATAAGA GAATTATATA TTTCTAACTA TATAACCCTA GGAATTTAGA    1844

CAACCTGAAA TTTATTCACA TATATCAAAG TGAGAAAATG CCTCAATTCA CATAGATTTC    1904

TTCTCTTTAG TATAATTGAC CTACTTTGGT AGTGGAATAG TGAATACTTA CTATAATTTG    1964

ACTTGAATAT GTAGCTCATC CTTTACACCA ACTCCTAATT TTAAATAATT TCTACTCTGT    2024

CTTAAATGAG AAGTACTTGG TTTTTTTTTT CTTAAATATG TATATGACAT TTAAATGTAA    2084

CTTATTATTT TTTTTGAGAC CGAGTCTTGC TCTGTTACCC AGGCTGGAGT GCAGTGGGTG    2144

ATCTTGGCTC ACTGCAAGCT CTGCCCTCCC CGGGTTCGCA CCATTCTCCT GCCTCAGCCT    2204
```

CCCAATTAGC TTGGCCTACA GTCATCTGCC ACCACACCTG GCTAATTTTT TGTACTTTTA 2264

GTAGAGACAG GGTTTCACCG TGTTAGCCAG GATGGTCTCG ATCTCCTGAC CTCGTGATCC 2324

GCCCACCTCG GCCTCCCAAA GTGCTGGGAT TACAGGCATG AGCCACCG 2372

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 491 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr
1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr
35 40 45

Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys
50 55 60

Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys Ser Asn Asp
65 70 75 80

Leu Leu Gly Asp Leu Phe Gly Val Pro Ser Phe Ser Val Lys Glu His
85 90 95

Arg Lys Ile Tyr Thr Met Ile Tyr Arg Asn Leu Val Val Val Asn Gln
100 105 110

Gln Glu Ser Ser Asp Ser Gly Thr Ser Val Ser Glu Asn Arg Cys His
115 120 125

Leu Glu Gly Gly Ser Asp Gln Lys Asp Leu Val Gln Glu Leu Gln Glu
130 135 140

Glu Lys Pro Ser Ser Ser His Leu Val Ser Arg Pro Ser Thr Ser Ser
145 150 155 160

Arg Arg Arg Ala Ile Ser Glu Thr Glu Glu Asn Ser Asp Glu Leu Ser
165 170 175

Gly Glu Arg Gln Arg Lys Arg His Lys Ser Asp Ser Ile Ser Leu Ser
180 185 190

Phe Asp Glu Ser Leu Ala Leu Cys Val Ile Arg Glu Ile Cys Cys Glu
195 200 205

Arg Ser Ser Ser Ser Glu Ser Thr Gly Thr Pro Ser Asn Pro Asp Leu
210 215 220

Asp Ala Gly Val Ser Glu His Ser Gly Asp Trp Leu Asp Gln Asp Ser
225 230 235 240

Val Ser Asp Gln Phe Ser Val Glu Phe Glu Val Glu Ser Leu Asp Ser
245 250 255

Glu Asp Tyr Ser Leu Ser Glu Glu Gly Gln Glu Leu Ser Asp Glu Asp
260 265 270

Asp Glu Val Tyr Gln Val Thr Val Tyr Gln Ala Gly Glu Ser Asp Thr
275 280 285

Asp Ser Phe Glu Glu Asp Pro Glu Ile Ser Leu Ala Asp Tyr Trp Lys
290 295 300

Cys Thr Ser Cys Asn Glu Met Asn Pro Pro Leu Pro Ser His Cys Asn
305 310 315 320

Arg Cys Trp Ala Leu Arg Glu Asn Trp Leu Pro Glu Asp Lys Gly Lys
325 330 335

Asp Lys Gly Glu Ile Ser Glu Lys Ala Lys Leu Glu Asn Ser Thr Gln
340 345 350

Ala Glu Glu Gly Phe Asp Val Pro Asp Cys Lys Lys Thr Ile Val Asn
355 360 365

Asp Ser Arg Glu Ser Cys Val Glu Glu Asn Asp Asp Lys Ile Thr Gln
370 375 380

Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro Ser Thr Ser
385 390 395 400

Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu Phe Glu Arg
405 410 415

Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser Leu Pro Leu
420 425 430

Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly
435 440 445

Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys Phe Thr Cys
450 455 460

Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln
465 470 475 480

Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro
485 490

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1710 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Mus musculus

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 202..1668

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT 60

CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC 120

TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGA CCCTCTCGGA TCACCGCGCT 180

TCTCCTGCGG CCTCCAGGCC A ATG TGC AAT ACC AAC ATG TCT GTG TCT ACC 231
Met Cys Asn Thr Asn Met Ser Val Ser Thr
1 5 10

GAG GGT GCT GCA AGC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACT 279
Glu Gly Ala Ala Ser Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr
15 20 25

```
CTG GTT AGA CCA AAA CCA TTG CTT TTG AAG TTG TTA AAG TCC GTT GGA      327
Leu Val Arg Pro Lys Pro Leu Leu Leu Lys Leu Leu Lys Ser Val Gly
            30                  35                  40

GCG CAA AAC GAC ACT TAC ACT ATG AAA GAG ATT ATA TTT TAT ATT GGC      375
Ala Gln Asn Asp Thr Tyr Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly
        45                  50                  55

CAG TAT ATT ATG ACT AAG AGG TTA TAT GAC GAG AAG CAG CAG CAC ATT      423
Gln Tyr Ile Met Thr Lys Arg Leu Tyr Asp Glu Lys Gln Gln His Ile
    60                  65                  70

GTG TAT TGT TCA AAT GAT CTC CTA GGA GAT GTG TTT GGA GTC CCG AGT      471
Val Tyr Cys Ser Asn Asp Leu Leu Gly Asp Val Phe Gly Val Pro Ser
75                  80                  85                  90

TTC TCT GTG AAG GAG CAC AGG AAA ATA TAT GCA ATG ATC TAC AGA AAT      519
Phe Ser Val Lys Glu His Arg Lys Ile Tyr Ala Met Ile Tyr Arg Asn
                95                  100                 105

TTA GTG GCT GTA AGT CAG CAA GAC TCT GGC ACA TCG CTG AGT GAG AGC      567
Leu Val Ala Val Ser Gln Gln Asp Ser Gly Thr Ser Leu Ser Glu Ser
            110                 115                 120

AGA CGT CAG CCT GAA GGT GGG AGT GAT CTG AAG GAT CCT TTG CAA GCG      615
Arg Arg Gln Pro Glu Gly Gly Ser Asp Leu Lys Asp Pro Leu Gln Ala
        125                 130                 135
```

CCA CCA GAA GAG AAA CCT TCA TCT TCT GAT TTA ATT TCT AGA CTG TCT 663
Pro Pro Glu Glu Lys Pro Ser Ser Ser Asp Leu Ile Ser Arg Leu Ser
140 145 150

ACC TCA TCT AGA AGG AGA TCC ATT AGT GAG ACA GAA GAG AAC ACA GAT 711
Thr Ser Ser Arg Arg Arg Ser Ile Ser Glu Thr Glu Glu Asn Thr Asp
155 160 165 170

GAG CTA CCT GGG GAG CGG CAC CGG AAG CGC CGC AGG TCC CTG TCC TTT 759
Glu Leu Pro Gly Glu Arg His Arg Lys Arg Arg Arg Ser Leu Ser Phe
175 180 185

GAT CCG AGC CTG GGT CTG TGT GAG CTG AGG GAG ATG TGC AGC GGC GGC 807
Asp Pro Ser Leu Gly Leu Cys Glu Leu Arg Glu Met Cys Ser Gly Gly
190 195 200

ACG AGC AGC AGT AGC AGC AGC AGC AGC GAG TCC ACA GAG ACG CCC TCG 855
Thr Ser Ser Ser Ser Ser Ser Ser Ser Glu Ser Thr Glu Thr Pro Ser
205 210 215

CAT CAG GAT CTT GAC GAT GGC GTA AGT GAG CAT TCT GGT GAT TGC CTG 903
His Gln Asp Leu Asp Asp Gly Val Ser Glu His Ser Gly Asp Cys Leu
220 225 230

GAT CAG GAT TCA GTT TCT GAT CAG TTT AGC GTG GAA TTT GAA GTT GAG 951
Asp Gln Asp Ser Val Ser Asp Gln Phe Ser Val Glu Phe Glu Val Glu
235 240 245 250

TCT CTG GAC TCG GAA GAT TAC AGC CTG AGT GAC GAA GGG CAC GAG CTC 999
Ser Leu Asp Ser Glu Asp Tyr Ser Leu Ser Asp Glu Gly His Glu Leu
255 260 265

TCA GAT GAG GAT GAT GAG GTC TAT CGG GTC ACA GTC TAT CAG ACA GGA 1047
Ser Asp Glu Asp Asp Glu Val Tyr Arg Val Thr Val Tyr Gln Thr Gly
270 275 280

GAA AGC GAT ACA GAC TCT TTT GAA GGA GAT CCT GAG ATT TCC TTA GCT 1095
Glu Ser Asp Thr Asp Ser Phe Glu Gly Asp Pro Glu Ile Ser Leu Ala
285 290 295

GAC TAT TGG AAG TGT ACC TCA TGC AAT GAA ATG AAT CCT CCC CTT CCA 1143
Asp Tyr Trp Lys Cys Thr Ser Cys Asn Glu Met Asn Pro Pro Leu Pro
300 305 310

TCA CAC TGC AAA AGA TGC TGG ACC CTT CGT GAG AAC TGG CTT CCA GAC 1191
Ser His Cys Lys Arg Cys Trp Thr Leu Arg Glu Asn Trp Leu Pro Asp
315 320 325 330

GAT AAG GGG AAA GAT AAA GTG GAA ATC TCT GAA AAA GCC AAA CTG GAA 1239
Asp Lys Gly Lys Asp Lys Val Glu Ile Ser Glu Lys Ala Lys Leu Glu
335 340 345

AAC TCA GCT CAG GCA GAA GAA GGC TTG GAT GTG CCT GAT GGC AAA AAG 1287
Asn Ser Ala Gln Ala Glu Glu Gly Leu Asp Val Pro Asp Gly Lys Lys
350 355 360

CTG ACA GAG AAT GAT GCT AAA GAG CCA TGT GCT GAG GAG GAC AGC GAG 1335
Leu Thr Glu Asn Asp Ala Lys Glu Pro Cys Ala Glu Glu Asp Ser Glu
365 370 375

GAG AAG GCC GAA CAG ACG CCC CTG TCC CAG GAG AGT GAC GAC TAT TCC 1383
Glu Lys Ala Glu Gln Thr Pro Leu Ser Gln Glu Ser Asp Asp Tyr Ser
380 385 390

CAA CCA TCG ACT TCC AGC AGC ATT GTT TAT AGC AGC CAA GAA AGC GTG 1431
Gln Pro Ser Thr Ser Ser Ser Ile Val Tyr Ser Ser Gln Glu Ser Val
395 400 405 410

AAA GAG TTG AAG GAG GAA ACG CAG CAC AAA GAC GAG AGT GTG GAA TCT 1479
Lys Glu Leu Lys Glu Glu Thr Gln His Lys Asp Glu Ser Val Glu Ser
415 420 425

AGC TTC TCC CTG AAT GCC ATC GAA CCA TGT GTG ATC TGC CAG GGG CGG 1527
Ser Phe Ser Leu Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg
430 435 440

CCT AAA AAT GGC TGC ATT GTT CAC GGC AAG ACT GGA CAC CTC ATG TCA 1575
Pro Lys Asn Gly Cys Ile Val His Gly Lys Thr Gly His Leu Met Ser
445 450 455

TGT TTC ACG TGT GCA AAG AAG CTA AAA AAA AGA AAC AAG CCC TGC CCA 1623
Cys Phe Thr Cys Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro
460 465 470

GTG TGC AGA CAG CCA ATC CAA ATG ATT GTG CTA AGT TAC TTC AAC 1668
Val Cys Arg Gln Pro Ile Gln Met Ile Val Leu Ser Tyr Phe Asn
475 480 485

TAGCTGACCT GCTCACAAAA ATAGAATTTT ATATTTCTAA CT 1710

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 489 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Cys Asn Thr Asn Met Ser Val Ser Thr Glu Gly Ala Ala Ser Thr
1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Asn Asp Thr Tyr
35 40 45

Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly Gln Tyr Ile Met Thr Lys
50 55 60

Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys Ser Asn Asp
65 70 75 80

Leu Leu Gly Asp Val Phe Gly Val Pro Ser Phe Ser Val Lys Glu His
85 90 95

Arg Lys Ile Tyr Ala Met Ile Tyr Arg Asn Leu Val Ala Val Ser Gln
100 105 110

Gln Asp Ser Gly Thr Ser Leu Ser Glu Ser Arg Arg Gln Pro Glu Gly
115 120 125

Gly Ser Asp Leu Lys Asp Pro Leu Gln Ala Pro Pro Glu Glu Lys Pro
130 135 140

Ser Ser Ser Asp Leu Ile Ser Arg Leu Ser Thr Ser Ser Arg Arg Arg
145 150 155 160

Ser Ile Ser Glu Thr Glu Glu Asn Thr Asp Glu Leu Pro Gly Glu Arg
165 170 175

His Arg Lys Arg Arg Arg Ser Leu Ser Phe Asp Pro Ser Leu Gly Leu
180 185 190

Cys Glu Leu Arg Glu Met Cys Ser Gly Gly Thr Ser Ser Ser Ser Ser
195 200 205

Ser Ser Ser Glu Ser Thr Glu Thr Pro Ser His Gln Asp Leu Asp Asp
210 215 220

Gly Val Ser Glu His Ser Gly Asp Cys Leu Asp Gln Asp Ser Val Ser
225 230 235 240

Asp Gln Phe Ser Val Glu Phe Glu Val Glu Ser Leu Asp Ser Glu Asp
245 250 255

Tyr Ser Leu Ser Asp Glu Gly His Glu Leu Ser Asp Glu Asp Asp Glu
260 265 270

Val Tyr Arg Val Thr Val Tyr Gln Thr Gly Glu Ser Asp Thr Asp Ser
275 280 285

Phe Glu Gly Asp Pro Glu Ile Ser Leu Ala Asp Tyr Trp Lys Cys Thr
290 295 300

Ser Cys Asn Glu Met Asn Pro Pro Leu Pro Ser His Cys Lys Arg Cys
305 310 315 320

Trp Thr Leu Arg Glu Asn Trp Leu Pro Asp Asp Lys Gly Lys Asp Lys
325 330 335

Val Glu Ile Ser Glu Lys Ala Lys Leu Glu Asn Ser Ala Gln Ala Glu
340 345 350

Glu Gly Leu Asp Val Pro Asp Gly Lys Lys Leu Thr Glu Asn Asp Ala
355 360 365

Lys Glu Pro Cys Ala Glu Glu Asp Ser Glu Glu Lys Ala Glu Gln Thr
370 375 380

Pro Leu Ser Gln Glu Ser Asp Asp Tyr Ser Gln Pro Ser Thr Ser Ser
385 390 395 400

Ser Ile Val Tyr Ser Ser Gln Glu Ser Val Lys Glu Leu Lys Glu Glu
405 410 415

Thr Gln His Lys Asp Glu Ser Val Glu Ser Ser Phe Ser Leu Asn Ala
420 425 430

Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly Cys Ile
435 440 445

Val His Gly Lys Thr Gly His Leu Met Ser Cys Phe Thr Cys Ala Lys
450 455 460

Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln Pro Ile
465 470 475 480

Gln Met Ile Val Leu Ser Tyr Phe Asn
485

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 10, line 19 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☒

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
March 11, 1993	HB 11290

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

Hybridoma: IF2

In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

M. Adams
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau is

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 10, line 19 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☒

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852, USA

Date of deposit	Accession Number
March 11, 1993	HB 11290

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

IF2 - Hybridoma

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

M. Holmes
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 10, line 19 of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet [ ] [2]

Name of depositary institution [3]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852, USA

Date of deposit [5]	Accession Number [6]
March 11, 1993	HB 11291

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

ED9 - Hybridoma

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

M. Holmes
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT RO/134 (January 1981)

CLAIMS

1. A method of diagnosing a neoplastic tissue in a human comprising:

detecting amplification of human MDM2 gene or elevated expression of a human MDM2 gene product in a tissue or body fluid isolated from a human, wherein amplification of the human MDM2 gene or elevated expression of human MDM2 gene product provides a diagnosis of neoplasia or the potential for neoplastic development.

2. The method of claim 1 wherein gene amplification is detected.

3. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being mRNA.

4. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being human MDM2 protein.

5. The method of claim 3 wherein said mRNA is detected by Northern blot analysis by hybridizing mRNA from said tissue to a human MDM2 nucleotide probe.

6. The method of claim 5 wherein the human MDM2 nucleotide probe comprises nucleotides 1-2372 of human MDM2, as shown in Figure 1, or fragments thereof consisting of at least 14 contiguous nucleotides.

7. The method of claim 4 wherein human MDM2 protein is detected by Western Blot analysis by reacting human MDM2 proteins with antibodies which are immunospecific for MDM2 protein.

8. The method of claim 2 wherein the gene amplification is detected using polymerase chain reaction.

9. The method of claim 2 wherein amplification of the human MDM2 gene is detected by Southern blot analysis wherein the human MDM2 gene is hybridized with a nucleotide probe which is complementary to hMDM2 DNA.

10. The method of claim 2 wherein gene amplification is determined by comparing the copy number of hMDM2 in the tissue to the copy number of hMDM2 in a normal tissue of the human.

11. The method of claim 3 wherein elevated expression of mRNA is determined by comparing the amount of hMDM2 mRNA in the tissue to the amount of hMDM2 mRNA in a normal tissue of the human.

12. The method of claim 4 wherein elevated expression of hMDM2 protein is determined by comparing the amount of hMDM2 protein in the tissue to the amount of hMDM2 protein in a normal tissue of the human.

13. The method of claim 2 wherein gene amplification is detected when at least 3-fold more hMDM-2 DNA is observed in the tissue relative to a control sample comprising a normal tissue.

14. The method of claim 3 wherein elevated expression is detected when at least 3-fold more hMDM-2 mRNA is observed in the tissue relative to a control sample comprising a normal tissue.

15. The method of claim 4 wherein elevated expression is detected when at least 3-fold more hMDM2 protein is observed in the tissue relative to a control sample comprising a normal tissue.

16. The method of claim 1 wherein the neoplasia is a sarcoma.

17. The method of claim 16 wherein the sarcoma is a liposarcoma, malignant fibrous histiocytoma, or osteosarcoma.

18. A cDNA molecule comprising nucleotides 1 to 2372, as shown in Figure 1, or fragments thereof, consisting of at least 14 contiguous nucleotides.

19. The cDNA molecule of claim 18 comprising the coding sequence of human MDM2.

20. Human MDM2 protein substantially free of other human proteins.

21. A preparation of antibodies specifically immunoreactive with human MDM2 protein.

22. The preparation of claim 21 wherein the antibodies are monoclonal antibodies.

23. A nucleotide probe comprising a sequence of at least 10 nucleotides which are complementary to nucleotides 1-2372 of human MDM2 gene, as shown in Figure 1.

24. A kit for detecting the amplification of a human MDM2 gene in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said human MDM2 gene under conditions of high stringency, and instructions for determining said amplification.

25. A kit for detecting elevated expression of a human MDM2 mRNA in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said mRNA, and written instructions for determining elevated expression of mRNA.

26. A kit for detecting elevated expression of a human MDM2 protein in a human tissue or body fluid sample comprising MDM2 protein-specific antibodies and written instructions for determining elevated expression of human MDM2 protein.

27. A method of treating a neoplastic cell or a cell having neoplastic potential, comprising:

administering to a cell a therapeutically effective amount of an inhibitory compound which interferes with the expression of human MDM2 gene.

28. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering antisense oligonucleotides.

29. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering triple-strand forming oligonucleotides which interact with DNA.

30. A method for identifying compounds which interfere with the binding of human MDM-2 to human p53, comprising:

binding a predetermined quantity of a first human protein which is detectably labelled to a second human protein;

adding a compound to be tested for its capacity to inhibit binding of said first and second proteins to each other;

determining the quantity of the first human protein which is displaced from or prevented from binding to the second human protein;

wherein the first human protein is MDM-2 and the second human protein is p53 or the first human protein is p53 and the second human protein is MDM-2.

31. The method of claim 30 wherein one of said two human proteins is fixed to a solid support.

32. The method of claim 30 wherein an antibody specifically immunoreactive with said second human protein is used to separate first human protein bound from unbound first human protein.

33. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering a polypeptide to tumor cells which contain a human MDM2 gene amplification, said polyptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

34. The method of claim 33 wherein said polypeptide comprises amino acids 1-41 of p53.

35. The method of claim 33 wherein said polypeptide comprises amino acids 13-57 of p53.

36. The method of claim 33 wherein said polypeptide comprises amino acids 1-50 of p53.

37. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

38. The method of claim 37 wherein said polypeptide comprises amino acids 1-41 of p53.

39. The method of claim 37 wherein said polypeptide comprises amino acids 13-57 of p53.

40. The method of claim 37 wherein said polypeptide comprises amino acids 1-50 of p53.

41. A polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide capable of binding to human MDM2.

42. The polypeptide of claim 41 which comprises amino acids 1-41 of p53.

43. The polypeptide of claim 41 which comprises amino acids 13-57 of p53.

44. The polypeptide of claim 41 which comprises amino acids 1-50 of p53.

45. The preparation of claim 21 wherein the antibodies do not bind to other human proteins.

46. The preparation of claim 21 wherein the antibodies do not bind to human proteins of M_r 75-85K, 105-120K, and 170-200K.

47. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).

48. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma ED9 (ATCC HB 11291).

49. The method of claim 7 wherein the antibodies bind to the epitope on hMDM2 bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).

50. The method of claim 4 wherein human MDM2 protein is detected by immunohistochemistry.

51. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by ED9 (ATCC HB 11291).

52. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by IF2 (ATCC HB 11290).

53. The method of claim 4 wherein human MDM2 protein is detected by immunoprecipitation.

54. A hybridoma cell having the identifying characteristics of ED9 (ATCC HB 11291).

55. A hybridoma cell having the identifying characteristics of IF2 (ATCC HB 11290).

FIG. 1A(1)

```
   1   GCACCGCGCGAGCTTGGCTGCTTCTGGGGC

                                     * AG
  84   GGCCGCGACCCCTCTGACCGAGATCCTGCTG

       CGT  GC  GG CTCCGCGCTCCCCG GAAG
 168   GTGCCCTGGCCCGGAGAGTGGAATGATCCCC

       ACC GACACCCCTGGGGGACC   TCG AT
 252   GGAGTCTTGAGGGACCCCCGACTCCAAGCGC
   1

       T    C  G        C  G
 336   CCTACTGATGGTGCTGTAACCACCTCACAGA
   9    P  T  D  G  A  V  T  T  S  Q
        S     E        A  S

         G         C     A  G     C
 420   TTATTAAAGTCTGTTGGTGCACAAAAAGACA
  37    L  L  K  S  V  G  A  Q  K  D
                                  N

       A G         C         G  G  C
 504   CGATTATATGATGAGAAGCAACAACATATTG
  65    R  L  Y  D  E  K  Q  Q  H  I

           G                     G A
 588   GTGAAAGAGCACAGGAAAATATATACCATGA
  93    V  K  E  H  R  K  I  Y  T  M
                                  A

         GC          G   AC    G C
 672   TCTGTGAGTGAGAACAGGTGTCACCTTGAAG
 121    S  V  S  E  N  R  C  H  L  E
           L        S     R  Q  P
```

FIG. 1A(2)

```
CTGTGTGGCCCTGTGTGTCGGAAAGATGGAGCAAGA

AGCCGC GC TTCTC TCG TCGAGCT TG ACGAC
CTTTCGCAGCCAGGAGCACCGTCCCTCCCCGGATTA

 GTCGGAA ATGCGC G AAGTAG    CC    T CT
GAGGCCCAGGGCGTCGTGCTTCCGCAGTAGTCAGTC

ACCGCG TTCTCCT C GCCTC      C
GAAAACCCCGGATGGTGAGGAGCAGGCAAATGTGCA
                                  M   C


                                T
TTCCAGCTTCGGAACAAGAGACCCTGGTTAGACCAA
I   P   A   S   E   Q   E   T   L   V   R   P


     C                  A   A A       A
CTTATACTATGAAAGAGGTTCTTTTTTATCTTGGCC
T   Y   T   M   K   E   V   L   F   Y   L   G
                        I   I           I

 G                          C           G
TATATTGTTCAAATGATCTTCTAGGAGATTTGTTTG
V   Y   C   S   N   D   L   L   G   D   L   F
                                        V

         A   T   A   G CT   A G         A----
TCTACAGGAACTTGGTAGTAGTCAATCAGCAGGAAT
I   Y   R   N   L   V   V   V   N   Q   Q   E
                        A       S           -

                TG      T C T G     C   CA
GTGGGAGTGATCAAAAGGACCTTGTACAAGAGCTTC
G   G   S   D   Q   K   D   L   V   Q   E   L
                L           P   L       A   P
```

FIG. 1A(3)

AGCCGAGCCCGAGGGGC	**83**	**Human nt**
CATG CGCTCA G C		Mouse nt
GTGCGTACGAGCGCCCA	**167**	**Human nt**
GGGCGAGC GAGACC		Mouse nt
CCCGTGAAGGAAACTGG	**251**	**Human nt**
G		Mouse nt
ATACCAACATGTCTGTA	**335**	**Human nt**
N T N M S V	**8**	**Human a.a.**
		Mouse a.a.
A		Mouse nt
AGCCATTGCTTTTGAAG	**419**	**Human nt**
K P L L L K	**36**	**Human a.a.**
		Mouse a.a.
G		Mouse nt
AGTATATTATGACTAAA	**503**	**Human nt**
Q Y I M T K	**64**	**Human a.a.**
		Mouse a.a.
A C G T		Mouse nt
GCGTGCCAAGCTTCTCT	**587**	**Human nt**
G V P S F S	**92**	**Human a.a.**
		Mouse a.a.
----- T C		Mouse nt
CATCGGACTCAGGTACA	**671**	**Human nt**
S S D S G T	**120**	**Human a.a.**
- -		Mouse a.a.
CA		Mouse nt
AGGAAGAGAAACCTTCA	**755**	**Human nt**
Q E E K P S	**148**	**Human a.a.**
P		Mouse a.a.

FIG. 1B(1)

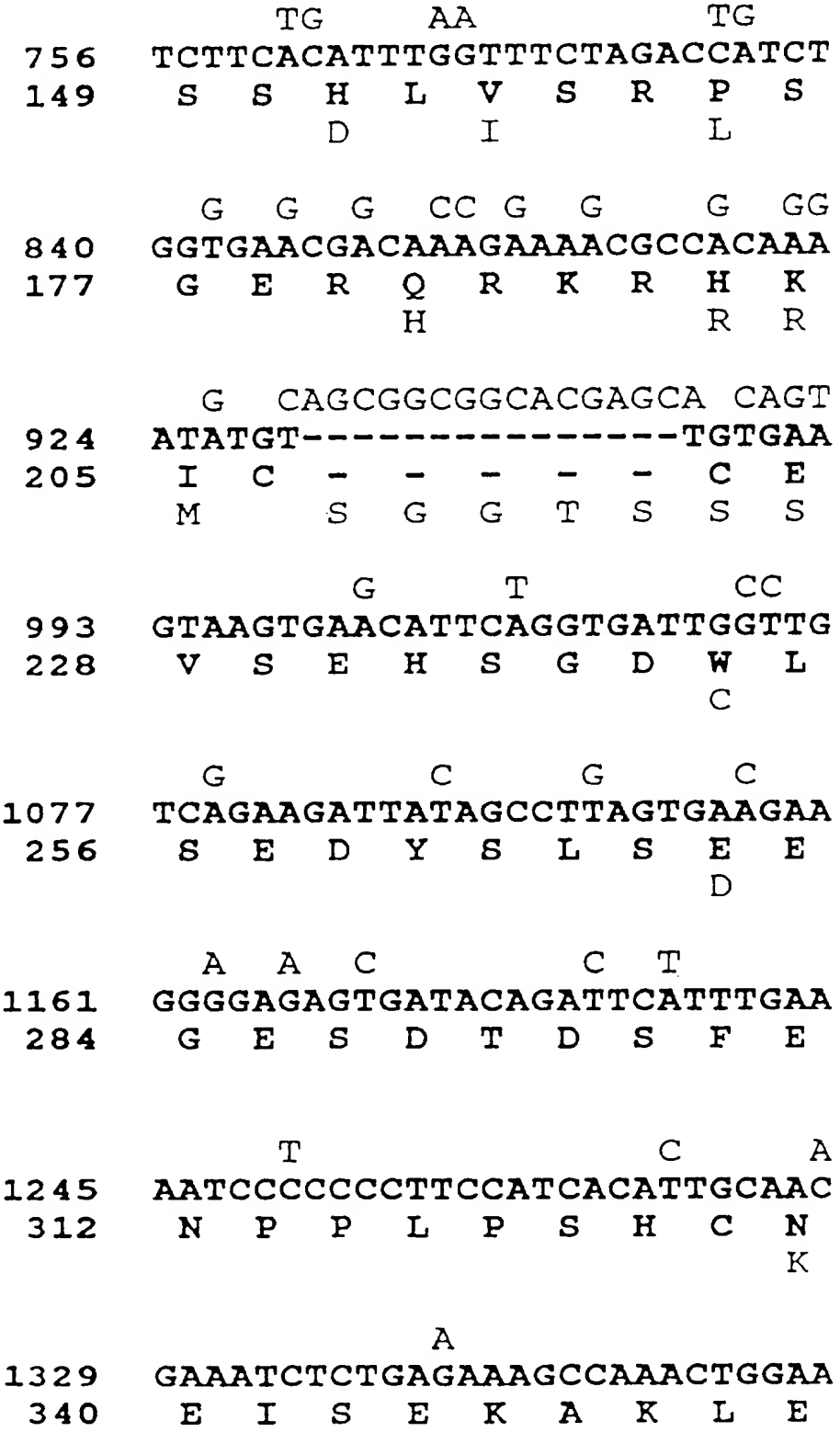

TG AA TG
756 TCTTCACATTTGGTTTCTAGACCATCT
149 S S H L V S R P S
D I L
G G G CC G G G GG
840 GGTGAACGACAAAGAAAACGCCACAAA
177 G E R Q R K R H K
H R R
G CAGCGGCGGCACGAGCA CAGT
924 ATATGT----------TGTGAA
205 I C - - - - - C E
M S G G T S S S
G T CC
993 GTAAGTGAACATTCAGGTGATTGGTTG
228 V S E H S G D W L
C
G C G C
1077 TCAGAAGATTATAGCCTTAGTGAAGAA
256 S E D Y S L S E E
D
A A C C T
1161 GGGGAGAGTGATACAGATTCATTTGAA
284 G E S D T D S F E
T C A
1245 AATCCCCCCCTTCCATCACATTGCAAC
312 N P P L P S H C N
K
A
1329 GAAATCTCTGAGAAAGCCAAACTGGAA
340 E I S E K A K L E

FIG. 1B(2)

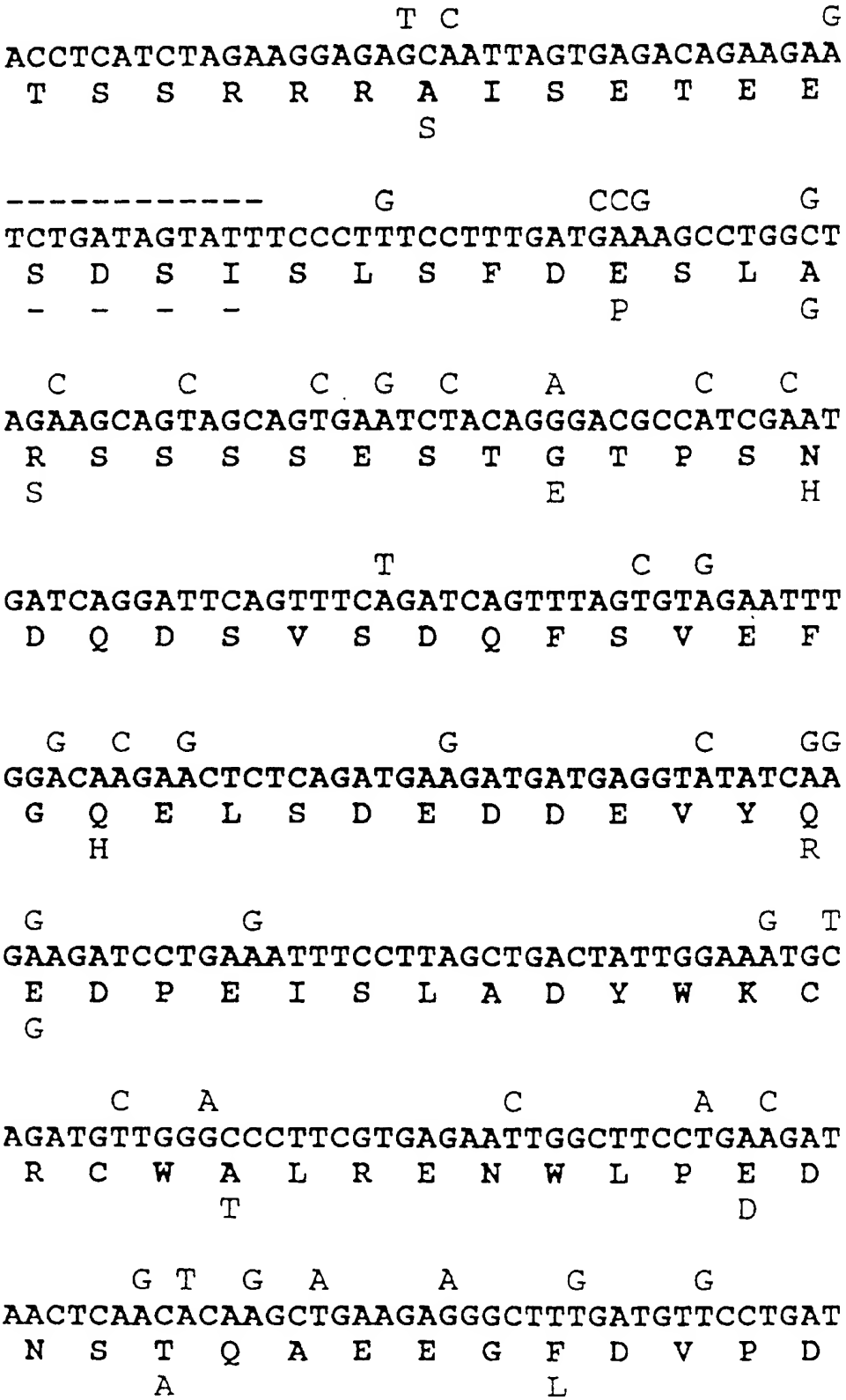

T C G
ACCTCATCTAGAAGGAGAGCAATTAGTGAGACAGAAGAA
T S S R R R A I S E T E E
S
---------- G CCG G
TCTGATAGTATTTCCCTTTCCTTTGATGAAAGCCTGGCT
S D S I S L S F D E S L A
- - - - P G
C C C G C A C C
AGAAGCAGTAGCAGTGAATCTACAGGGACGCCATCGAAT
R S S S S E S T G T P S N
S E H
T C G
GATCAGGATTCAGTTTCAGATCAGTTTAGTGTAGAATTT
D Q D S V S D Q F S V E F
G C G G C GG
GGACAAGAACTCTCAGATGAAGATGATGAGGTATATCAA
G Q E L S D E D D E V Y Q
H R
G G G T
GAAGATCCTGAAATTTCCTTAGCTGACTATTGGAAATGC
E D P E I S L A D Y W K C
G
C A C A C
AGATGTTGGGCCCTTCGTGAGAATTGGCTTCCTGAAGAT
R C W A L R E N W L P E D
T D
G T G A A G G
AACTCAACACAAGCTGAAGAGGGCTTTGATGTTCCTGAT
N S T Q A E E G F D V P D
A L

FIG. 1B(3)

```
  CA         GC  C                    Mouse nt
AATTCAGATGAATTATCT      839           Human nt
 N  S  D  E  L  S       176           Human a.a.
    T            P                   Mouse a.a.

       AGC G                          Mouse nt
CTGTGTGTAATAAGGGAG      923           Human nt
 L  C  V  I  R  E       204           Human a.a.
       E  L                           Mouse a.a.

 A          C A  C                    Mouse nt
CCGGATCTTGATGCTGGT      992           Human nt
 P  D  L  D  A  G       227           Human a.a.
 Q           D                        Mouse a.a.

        G     G                       Mouse nt
GAAGTTGAATCTCTCGAC     1076           Human nt
 E  V  E  S  L  D       255           Human a.a.
                                      Mouse a.a.

  C  A  C       A                     Mouse nt
GTTACTGTGTATCAGGCA     1160           Human nt
 V  T  V  Y  Q  A       283           Human a.a.
                T                     Mouse a.a.

  C                                   Mouse nt
ACTTCATGCAATGAAATG     1244           Human nt
 T  S  C  N  E  M       311           Human a.a.
                                      Mouse a.a.

  G                T                  Mouse nt
AAAGGGAAAGATAAAGGG     1228           Human nt
 K  G  K  D  K  G       339           Human a.a.
                V                     Mouse a.a.

G C     GCTG C  A                     Mouse nt
TGTAAAAAAACTATAGTG     1412           Human nt
 C  K  K  T  I  V       367           Human a.a.
 G        L  T  E                     Mouse a.a.
```

FIG. IC(1)

```
                G T A     C       C       G
1413  AATGATTCCAGAGAGTCATGTGTTGAGGAA
 368    N  D  S  R  E  S  C  V  E  E
          A  K     P     A

        C  A     G     C  C       G
1494  TCTCAGCCATCAACTTCTAGTAGCATTATT
 395    S  Q  P  S  T  S  S  S  I  I
                                    V

        C                      C  CT    G
1578  GA? ?GAGTGTGGAATCTAGTTTGCCCCTT
 423    E     S  V  E  S  S  L  P  L
        D                      F  S

        T  ?     G  T     C  C   T A
1662  GTCCAT?GCAAAACAGGACATCTTATGGCC
 451    V  H  G  K  T  G  H  L  M  A
                                    S

           G     C                  G
1746  AGACAACCAATTCAAATGATTGTGCTAACT
 479    R  Q  P  I  Q  M  I  V  L  T
                                    S

1830  TAACCCTAGGAATTTAGACAACCTGAAATT
1914  TTAGTATAATTGACCTACTTTGGTAGTGGA
1998  ACTCCTAATTTTAAATAATTTCTACTCTGT
2082  ATGTAACTTATTATTTTTTTTGAGACCGAG
2166  CTCTGCCCTCCCCGGGTTCGCACCATTCTC
2250  TAATTTTTTGTACTTTTAGTAGAGACAGGG
2334  CTCGGCCTCCCAAAGTGCTGGGATTACAGG
```

FIG. 1C(2)

```
G CAGC  G  G  GGCCGA   GA GC C TG  C
AAT---GATGATAAAATTACACAAGCTTCACAATCAC
 N  -  D  D  K  I  T  Q  A  S  Q  S
 D  S  E  E     A  E     T  P  L

                  AGC            G--- A
TATAGCAGCCAAGAAGATGTGAAAGAGTTTGAAAGGG
 Y  S  S  Q  E  D  V  K  E  F  E  R
                  S           L  -  K

         C     A          C  C  G  G  G
AATGCCATTGAACCTTGTGTGATTTGTCAAGGTCGAC
 N  A  I  E  P  C  V  I  C  Q  G  R

  T  C  G                       A     A  C
TGCTTTACATGTGCAAAGAAGCTAAAGAAAAGGAATA
 C  F  T  C  A  K  K  L  K  K  R  N

  C   AA    C        CTCA A A  T
TATTTCCCCTAGTTGACCTG---TCTATAAGAGAATT
 Y  F  P
       N

TATTCACATATATCAAAGTGAGAAAATGCCTCAATTC
ATAGTGAATACTTACTATAATTTGACTTGAATATGTA
CTTAAATGAGAAGTACTTGGTTTTTTTTTTCTTAAAT
TCTTGCTCTGTTACCCAGGCTGGAGTGCAGTGGGTGA
CTGCCTCAGCCTCCCAATTAGCTTGGCCTACAGTCAT
TTTCACCGTGTTAGCCAGGATGGTCTCGATCTCCTGA
CATGAGCCACCG
```

FIG. 1C(3)

```
 G  G     C                          Mouse nt
AAGAAAGTGAAGACTAT      1493          Human nt
Q  E  S  E  D  Y        394          Human a.a.
         D                           Mouse a.a.

 G     G  GC                         Mouse nt
AAGAAACCCAAGACAAA      1577          Human nt
E  E  T  Q  D  K        422          Human a.a.
            H                        Mouse a.a.

            C                        Mouse nt
CTAAAAATGGTTGCATT      1661          Human nt
P  K  N  G  C  I        450          Human a.a.
                                     Mouse a.a.

               G  C                  Mouse nt
AGCCCTGCCCAGTATGT      1745          Human nt
K  P  C  P  V  C        478          Human a.a.
                                     Mouse a.a.

T              *                     Mouse nt
ATATATTTCTAACTATA       1829         Human nt
                         491         Human a.a.
                                     Mouse a.a.

ACATAGATTTCTTCTCT       1913         Human nt
GCTCATCCTTTACACCA       1997         Human nt
ATGTATATGACATTTAA       2081         Human nt
TCTTGGCTCACTGCAAG       2165         Human nt
CTGCCACCACACCTGGC       2249         Human nt
CCTCGTGATCCGCCCAC       2333         Human nt
                        2372         Human nt
```

FIG. 2

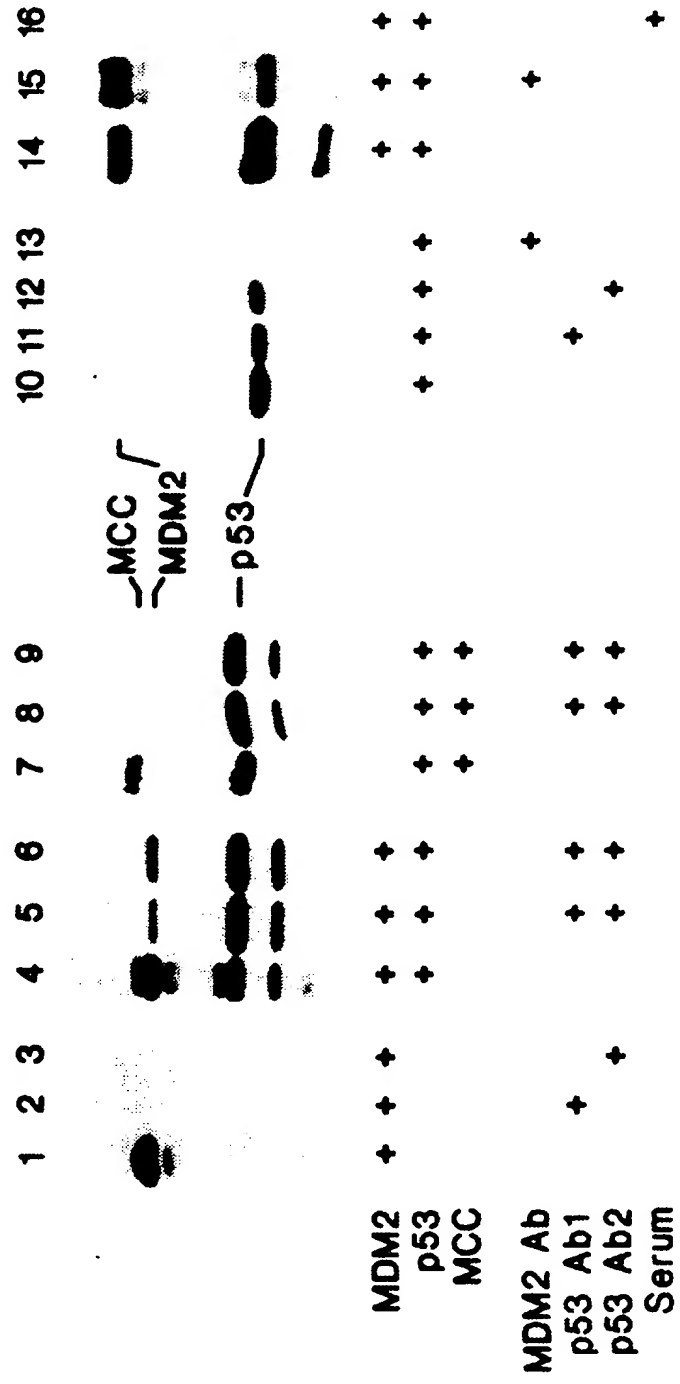

16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
MCC
MDM2
p53
MDM2
p53
MCC
MDM2 Ab
p53 Ab1
p53 Ab2
Serum

FIG. 3

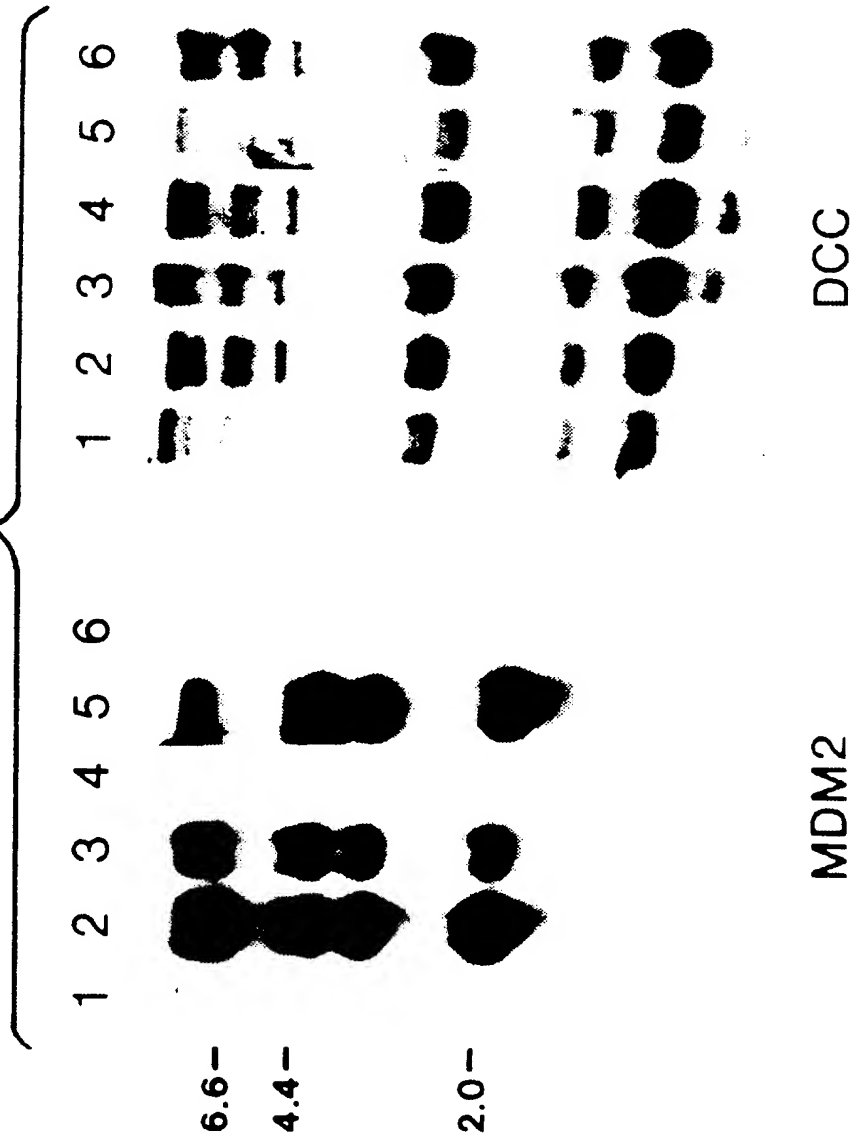


FIG. 4A

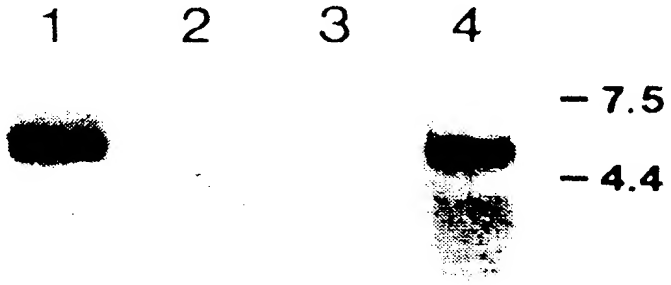


FIG. 4B

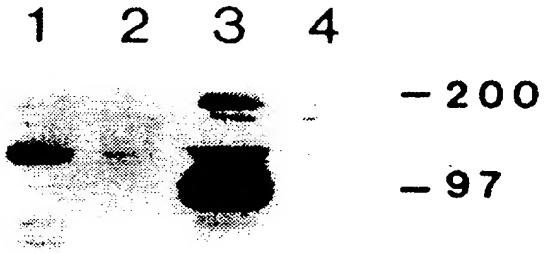


FIG. 4C

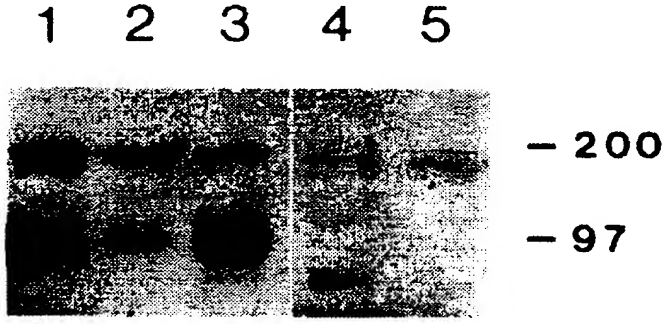
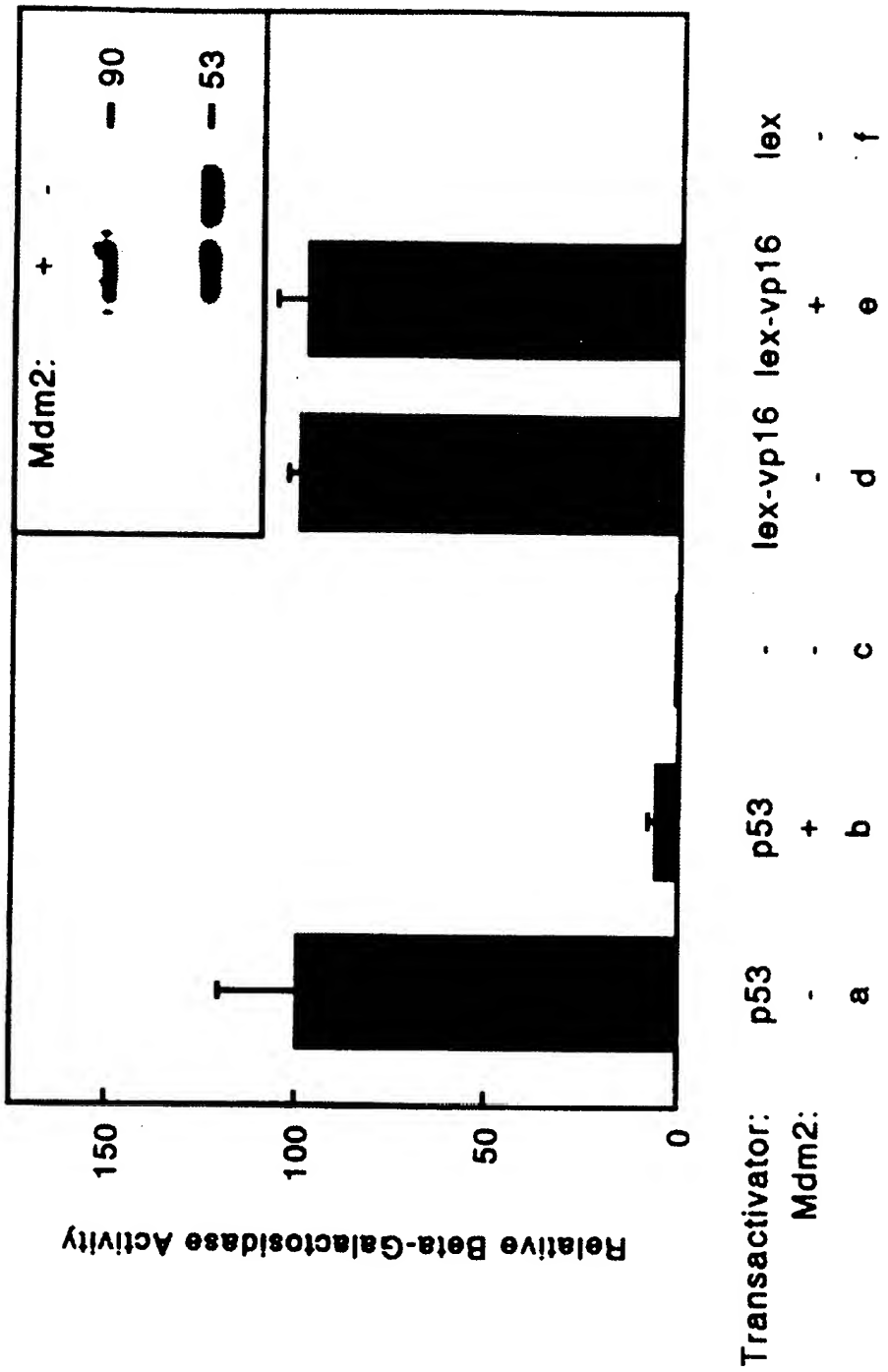


FIG. 5


Relative Beta-Galactosidase Activity
150
100
50
0
Mdm2: + -
90
53
Transactivator: p53 p53 - lex-vp16 lex-vp16 lex
Mdm2: - + - - + -
a b c d e f

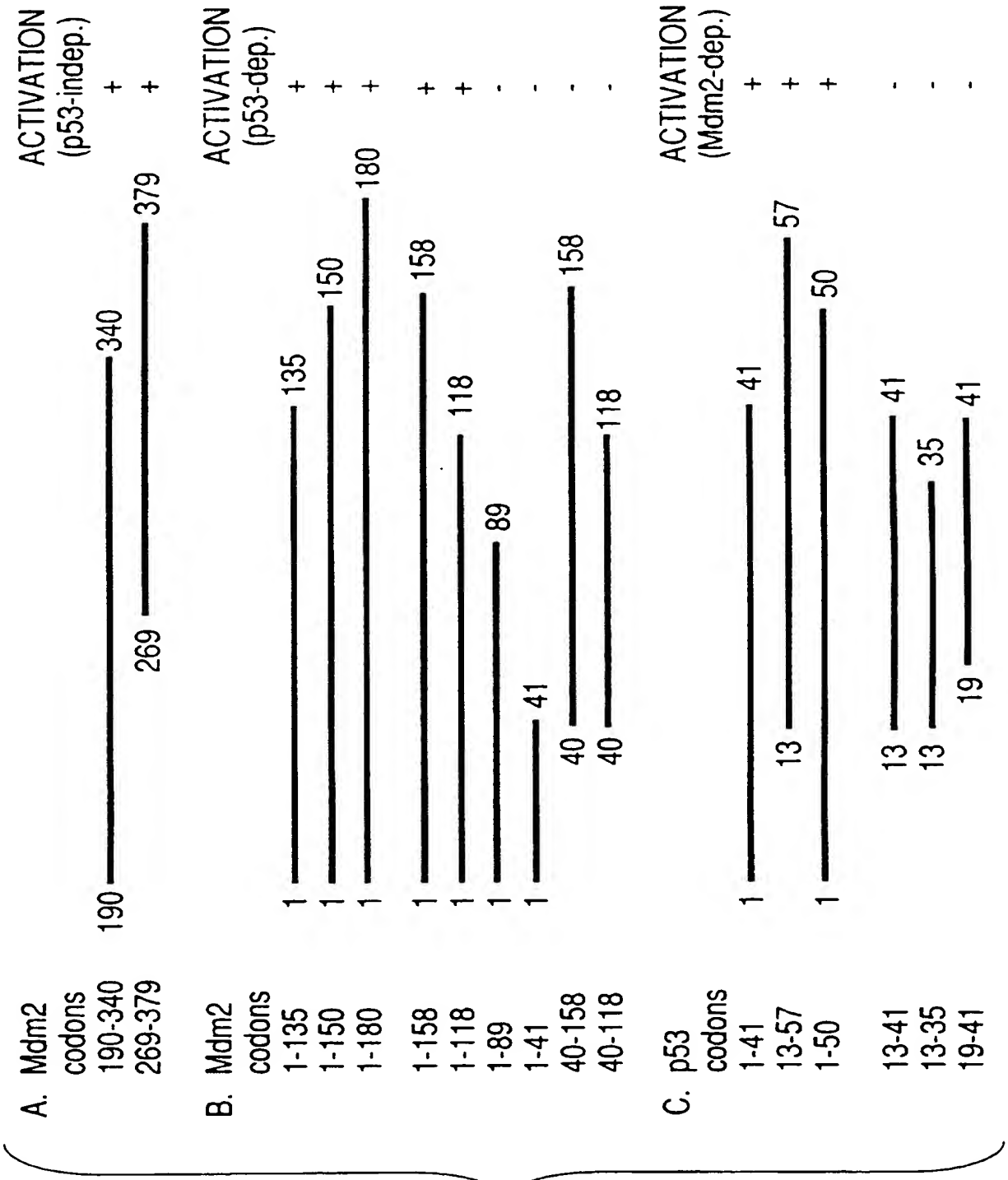

A. Mdm2 codons
190-340
269-379
ACTIVATION (p53-indep.)
+
+
190
340
269
379
B. Mdm2 codons
1-135
1-150
1-180
1-158
1-118
1-89
1-41
40-158
40-118
ACTIVATION (p53-dep.)
+
+
+
+
+
-
-
-
1
135
150
180
158
118
89
41
40
158
40
118
C. p53 codons
1-41
13-57
1-50
13-41
13-35
19-41
ACTIVATION (Mdm2-dep.)
+
+
+
-
-
-
1
41
13
57
1
50
13
41
13
35
19
41

FIG. 6

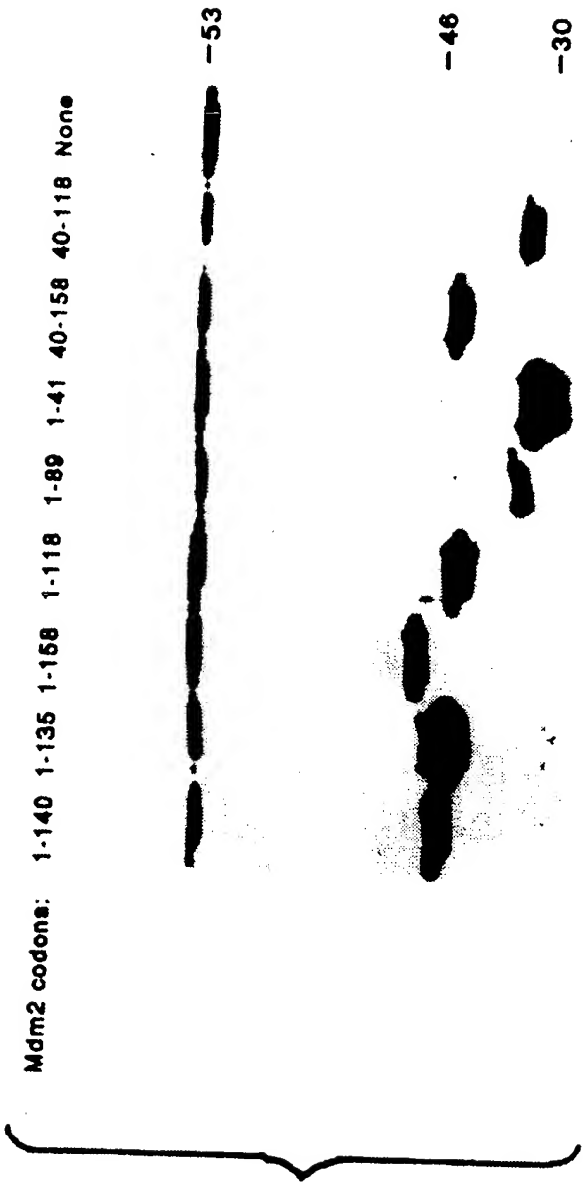

Mdm2 codons: 1-140 1-135 1-158 1-118 1-89 1-41 40-158 40-118 None
-53
-46
-30

FIG. 7A

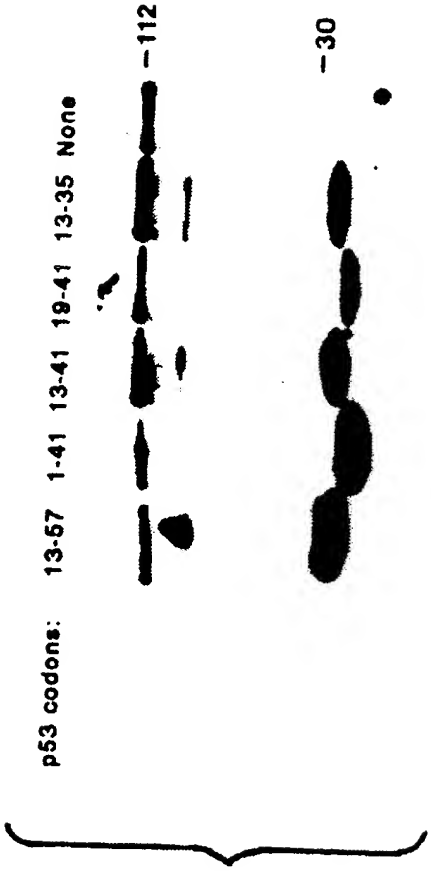

p53 codons: 13-57 1-41 13-41 19-41 13-35 None
-112
-30

FIG. 7B

FIG. 8

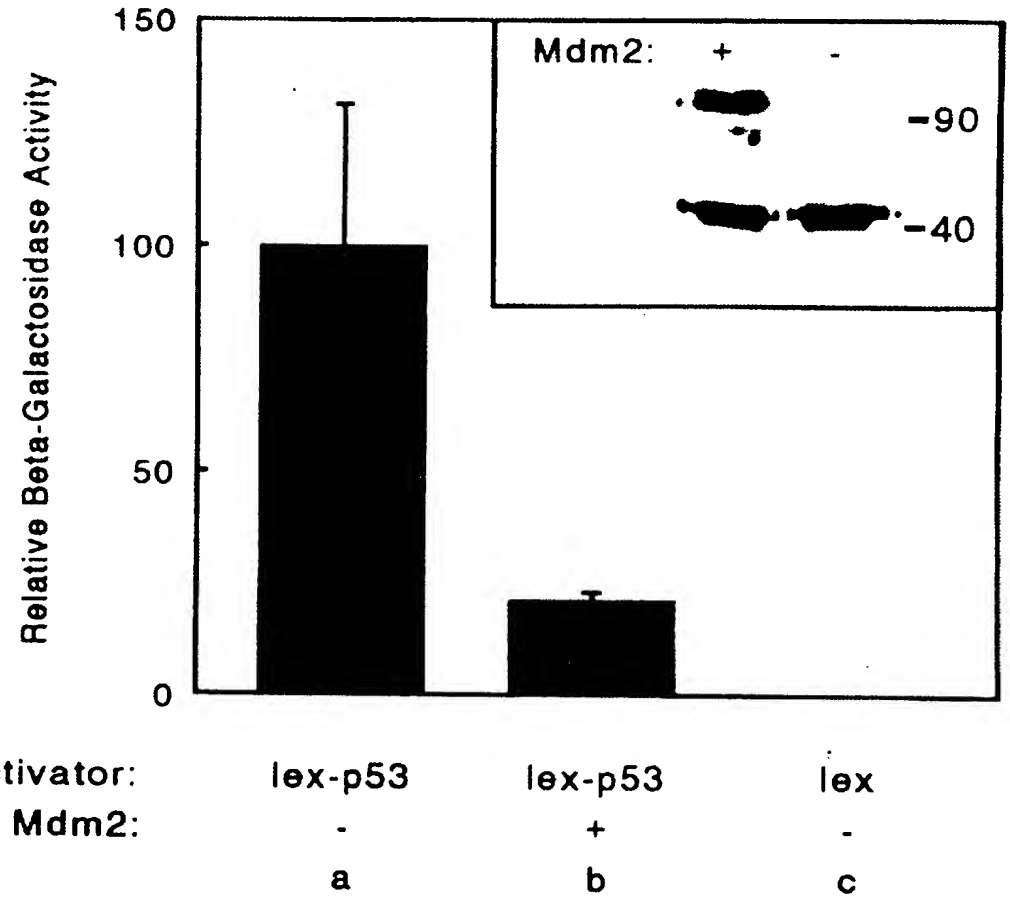

Relative Beta-Galactosidase Activity
150
100
50
0
Mdm2: + -
-90
-40
Transactivator: lex-p53 lex-p53 lex
Mdm2: - + -
a b c

FIGURE 9

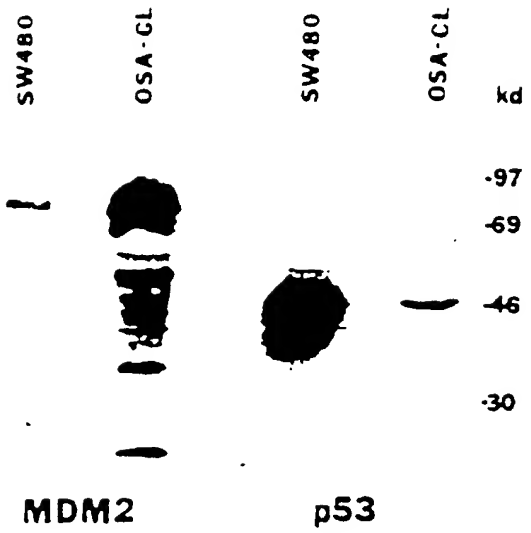


FIGURE 10

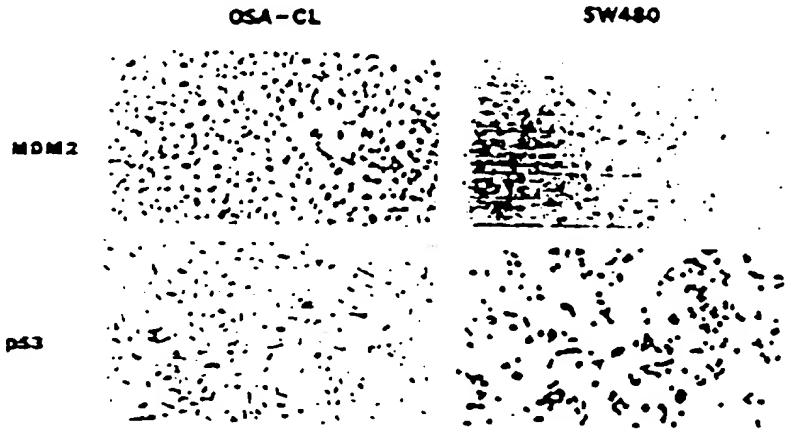


FIGURE 11

INTERNATIONAL SEARCH REPORT

International Application N PCT/US 93/03199

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; G01N33/574; C07H21/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	EMBO JOURNAL. vol. 10, no. 6, 1991, EYNSHAM, OXFORD GB pages 1565 - 1569 FAKHARZADEH ET AL. 'Tumoriginic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line' cited in the application see the whole document ---	1-55
A	J BIOL CHEM 263 (32). 1988. 17150-17158. SNYDER L C ET AL. 'A GENE AMPLIFIED IN A TRANSFORMED MOUSE CELL LINE UNDERGOES COMPLEX TRANSCRIPTIONAL PROCESSING AND ENCODES A NUCLEAR PROTEIN.' ---	-

-/--

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 SEPTEMBER 1993	01. 10. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MOLINA GALAN E.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CELL GROWTH & DIFFERENTIATION vol. 1, 1990, pages 571 - 580 HINDS ET AL. 'Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes' cited in the application	-
A	EP,A,0 341 904 (TEMPLE UNIVERSITY) 15 November 1989 see abstract	30-33
A	US,A,4 968 603 (SLAMON ET AL.) 6 November 1990	-
P,X	NATURE vol. 358, 2 July 1992, LONDON GB pages 80 - 83 OLINER ET AL. 'Amplification of a gene encoding a p53 associated protein in human sarcomas' see the whole document	1,18,20
P,X	CELL 69 (7). 1992. 1237-1245. MOMAND J ET AL. 'THE MDM -2 ONCOGENE PRODUCT FORMS A COMPLEX WITH THE'	20

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9303199
SA 73548

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 17/09/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0341904	15-11-89	JP-A- 2013400	17-01-90
US-A-4968603	06-11-90	None	